# Stress-Induced Oviposition Delays in Laying Hens and their Physiological Causation

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# Declaration

I declare that the work described in this thesis is my own composition. The work presented in it was conducted by myself, with the exception of the operations described in Chapter 5 which were performed by Dr. C.J.Savory, and has not been presented in any other thesis. All help and assistance received has been acknowledged.

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# Abstract

A variety of stressors cause hens to delay oviposition and this can result in characteristic eggshell abnormalities which are commercially undesirable. This project examined the duration of stress-induced oviposition delays in medium-hybrid hens and investigated their underlying physiological causation. Social stress (relocation of hens from individual to group cages) was used to induce the oviposition delays.

From behavioural observations of stress-induced oviposition delays, it was found that eggs were laid either before a threshold of approximately 3 h delay or after at least 7 h delay. This distinction between short- and long-term delays accounts for different types of eggshell abnormality.

Oviposition is caused by uterine contractions induced by release into the circulation of ovarian prostaglandins and neurohypophysial arginine vasotocin (AVT). Two possible physiological mechanisms which may account for the stress-induced oviposition delays were examined. First, opioid peptides released in response to stress may inhibit AVT release. Second, adrenaline released in response to stress may directly suppress uterine contractions.

Plasma AVT concentration was elevated at the time of delayed oviposition, but not during the delay, and AVT injection rapidly ended delays, thus supporting the hypothesis that delays are caused by inhibition of AVT release. The possible role of opioid release in causation of stress-induced oviposition delays was investigated by treating hens with appropriate receptor antagonist and agonist compounds. Nalmefene, a non-specific opioid receptor antagonist, significantly reduced the duration of stress-induced oviposition delays. U50,488, a selective kappa receptor agonist, induced oviposition delays, an effect prevented by nalmefene and ended by AVT injection. U50,488 also increased the time from prostaglandin-F2$\alpha$ injection to premature oviposition induction, and reduced the associated rise in plasma AVT concentration. Morphine, a selective mu receptor agonist, induced some ovipositions prematurely but may have delayed others. These findings support the (first) hypothesis that opioids delay oviposition by inhibiting AVT release. The frequency of headflicking in response to alerting stimuli, a putative index of hens' opioid status, was significantly reduced by social stress. This evidence is consistent with the assumption that opioids are released during social stress.

Propranolol, a $\beta$-adrenergic receptor blocker, prevented stress-induced oviposition delays, supporting the (second) hypothesis that these are caused by adrenergic suppression of uterine contractions. Thus, the mechanisms underlying stress-induced oviposition delays may involve both endogenous opioids, which inhibit AVT release, and adrenaline, which directly suppresses uterine contractions.

As oviposition is associated with ovulation of the subsequent egg, it was of interest to establish whether stress-induced oviposition delays were associated with delays of the subsequent eggs ovulation. From investigations of the stage of formation and position of the subsequent egg in the oviduct, it was concluded that stress can cause short ovulation delays.

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# CHAPTER 1: **General Introduction**

A variety of stressors cause hens to delay oviposition and this can result in characteristic eggshell abnormalities which are commercially undesirable. This thesis examines the duration of stress-induced oviposition delays in medium-hybrid hens and investigates their underlying physiological causation.

In this Introduction, the production of eggs and their shells by hens is summarised first (Section 1.1). The literature relating to stress-induced oviposition delays is reviewed in Section 1.2 and then the consequences of these delays for eggshell quality are described in Section 1.3. Physiological control of oviposition is described in Section 1.4, then ways in which stress might disrupt this to delay oviposition are hypothesised in Section 1.5. The possible roles of opioid peptides and adrenaline in the mechanisms underlying stress-induced oviposition delays are introduced in Sections 1.6 and 1.7. Finally, Section 1.8 outlines the experimental approach followed in this thesis.

## 1.1 Egg Production by Laying Hens

There are two types of domestic hen used by the poultry industry: laying hens, such as ISA Brown and Hisex, which have been selected for high egg yield, and meat type chickens, broilers, which have been selected for high meat yield. Hens of the modern egg laying strains examined in this thesis lay almost every day once mature and individuals are expected to lay over 300 eggs in their productive year (Appleby *et al.,* 1992). This section summarises the daily cycle of egg laying and the structure and formation of eggshells. For further information on the egg laying cycle the reader is referred to reviews by Johnson (1986), Etches (1990) and Bahr and Johnson (1991). The structure and formation of eggshells is reviewed by Solomon (1991) and Roberts and Brackpool (1994).

The hen's reproductive system is comprised of a single ovary and a single oviduct which is divided into functionally distinct regions, each concerned with different aspects of egg formation. These organs and an indication of the time taken for an egg to pass through each of the oviduct regions are shown Figure 1.1.

The ovary contains a large number of developing ova arranged in a follicular hierarchy from which the largest is due to ovulate next. Progesterone released into the circulation by the largest pre-ovulatory follicle stimulates the anterior pituitary to

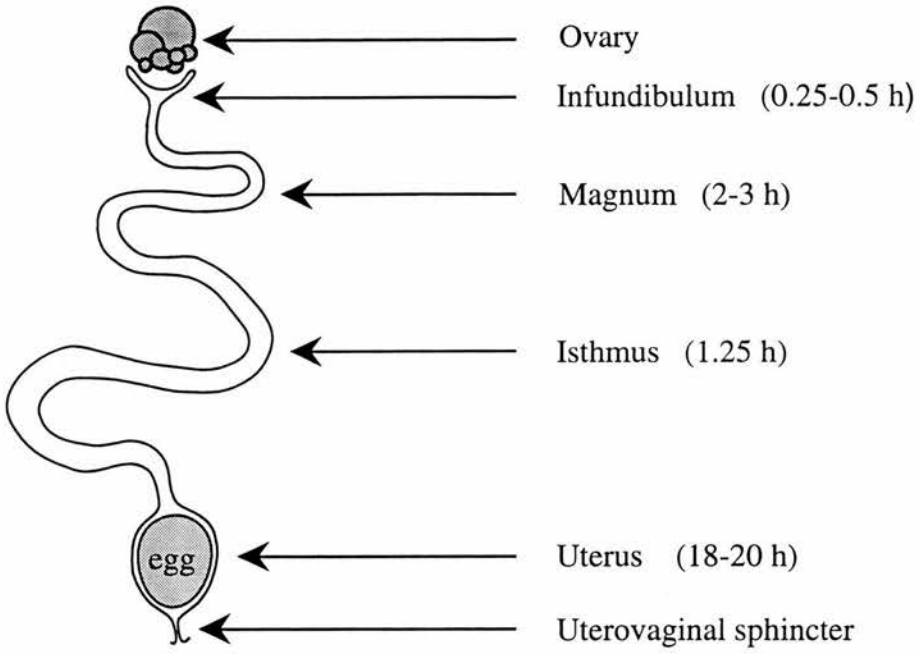



**Figure 1.1** A diagrammatic representation of the reproductive organs of the laying hen, with an egg in the uterus. The approximate times taken for the developing egg to pass through each oviduct section are shown in brackets (from Warren & Scott, 1935).

release lutenising hormone (LH), which then causes this follicle to ovulate about 5 h later. The light-dark cycle affects the release of LH in such a way that ovulation is restricted to an 8 h period in the morning (Etches, 1984). As oviposition occurs 24-25 h after ovulation, oviposition is similarly restricted and most occur in the 12-18 h after dusk, i.e. in the morning when conventional lighting patterns (14L:10D) are used. Ruptured follicles from recent ovulations (post-ovulatory follicles) are also present in the ovary and are involved in endocrine control of pre-laying behaviours and possibly also timing of oviposition.

Once ovulated, the ovum is engulfed by the infundibulum and so enters the oviduct. Occasionally the ovulated ovum is not picked up by the infundibulum and remains in the body cavity where it is eventually reabsorbed. This is described as 'internal ovulation' and results in a day when no egg is laid (Wood-Gush & Gilbert, 1970). After the infundibulum, the egg passes through the magnum, where the albumen layer is laid down, and then through the isthmus where the shell membrane is formed. Finally, 4-5 h after ovulation, the egg enters the uterus (shell gland) where eggshell formation occurs. Oviposition, the expulsion of the egg from the

uterus through the uterovaginal sphincter, takes place 24-25 h after ovulation and is accompanied by characteristic pre-laying behaviour patterns. The next day's egg is usually ovulated 15-45 min after oviposition and the process of egg formation starts again.

A group of eggs laid on consecutive days is described as a sequence. Sequences are separated by days on which there is no oviposition. Oviposition time is earliest for the first egg of a sequence, and is latest for the last egg. The change in oviposition time between two consecutive days, the lag, is greatest at the start and end of sequences and may be effectively zero in the middle of long sequences. This pattern of oviposition times is a consequence of circadian rhythms which restrict the period in which LH may be released and thus the times when ovulation can occur (Etches, 1984). Examples of sequences of various lengths can be found in Lillpers and Wilhelmson (1993b).

Oviposition is preceded by characteristic behaviour patterns associated with the finding and preparation of a suitable nest site, and which are divided into two phases: a searching phase and a nesting phase (Freire, 1994). During the searching phase, cage-housed hens typically show restlessness and frantic pacing, while the nesting phase is characterised by sitting and sometimes vigorous attempts to find cover. Pre-laying behaviour is not controlled by the presence of an egg in the oviduct but instead by the post-ovulatory follicles in the ovary, in particular that which ovulated 24 h previously (Wood-Gush & Gilbert, 1964). Consequently pre-laying behaviour occurs approximately 24 h after an internal ovulation even though there is no egg to be laid (Wood-Gush, 1963). Food intake and gut motility are suppressed during pre-laying behaviour but resume shortly after oviposition (Savory, 1977; Shimada, 1986; Shimada *et al.*, 1987b).

Eggshells have two important functions: they protect the developing embryos and, from a commercial perspective, provide packaging for their food contents. In their capacity as incubation chambers eggshells must, in addition to providing the embryo with physical protection and a source of calcium, allow movement of respiratory gases. Artificial genetic selection has been used to improve the colour and strength of eggshells.

The shell membrane is laid down as the egg passes through the isthmus and becomes the foundation for the eggshell, providing centres for initiation of calcite crystallisation. During the 18-20 h the egg spends in the uterus, calcium carbonate is laid down, forming characteristic layers which provide the shell with most of its strength. Under hormonal control linked to the egg's own ovulation, calcium

mobilised from the skeleton and from increased intestinal absorption is transported to the uterus in the blood. It is ovulation and not the presence of an egg in the uterus that stimulates eggshell formation (Eastin & Spaziani, 1978). Finally, about 1.5 h before oviposition, calcification stops and the cuticle is laid down. The cuticle is the outermost layer of the eggshell and is an organic matrix that contains most of the shell's pigmentation. It protects the egg from water penetration, water loss and microbial invasion.

Some eggs carry an additional layer of calcification overlying the cuticle which is visible as a chalky coating on the surface of brown eggshells. This feature, described as eggshell dusting, is most common early in lay and may be unevenly distributed over the eggshell (Mills *et al.*, 1991).

## 1.2 Stress-Induced Oviposition Delays

It is known that environmental stress can cause hens to delay the time at which their ovipositions occur. In one of the earliest reports, Patterson (1909) describes how ovipositions were delayed for up to 20 h by disturbing hens every time they attempted to lay. Scott (1940) observed that hens often delayed oviposition when they were moved to unfamiliar environments shortly before they were due to lay. Quail can also be induced to delay oviposition, for 1-28 h and sometimes even longer, by frightening procedures such as dropping a metal drum onto a hard surface (Opel, 1966).

The presence of unfamiliar birds and alterations to social group composition also induce oviposition delays. Hughes (1979) found that replacing one hen from an established group of four with an unfamiliar hen shortly before they were due to lay increased the amount of aggressive behaviour and caused oviposition delays. The replacement hens delayed oviposition longer than did the residents. Similarly, Watt and Solomon (1988) and Watt (1989) reported that moving six individually housed hens into a single cage shortly before they were due to lay also delayed oviposition.

Before oviposition time hens become highly motivated to find a suitable nest site in which to lay their egg. Duncan (1970) reported that when hens which were housed in deep litter pens and were used to laying in nest boxes were deprived of access to these nest sites, either by closing the nests or by moving the birds to cages, ovipositions were delayed. Moving hens to cages caused oviposition delays that were, on average, 1 h longer than those caused by nest box closure, presumably because relocation was an additional source of stress. Kite (1985) reported that

some hens would regularly delay oviposition for over 24 h when deprived of a nest site and would lay within a few minutes of being provided with a nest box. Hughes *et al.* (1986) also found that excluding hens from regularly used nest sites induced oviposition delays which often exceeded 13 h; they described how pre-laying behaviours were present during the first few hours of the oviposition delay, but then became interspersed with and finally replaced by other behaviours. Cooper and Appleby (1994) found that partially obstructing access to a nest site, leaving only a narrow gap through which the hen could pass with difficulty, delayed oviposition for 1-3 h.

It is not clear if motivational states such as hunger or thirst, which may conflict with nesting motivation, can cause oviposition delays. Although the distribution of oviposition times throughout the day may be influenced by daily feeding time (Brake, 1985; Wilson & Keeling, 1991), it is not clear if these effects are due to the food itself or the general disturbance associated with the food presentation. Freire (1994) and Appleby and Freire (1995) found that the nesting behaviour of hens housed in a row of cages was temporarily replaced by food directed activity when their food was topped up at a time when most were soon due to lay. Hens receiving this food within 15 min of the time when their oviposition was due delayed oviposition for 9-42 min, and this was interpreted in terms of conflicting motivations for feeding and egg laying.

From an evolutionary point of view, delaying oviposition in response to stresses such as the absence of a nest site should ensure that birds do not lay their eggs in places unsuitable for incubation. Furthermore, birds in natural habitats may be vulnerable to predation during oviposition, and so delaying this process when danger threatens would be a selective advantage.

## 1.3 Eggshell Abnormalities Associated with Oviposition Delays

### 1.3.1 Causation and Classification of Eggshell Abnormalities

When oviposition is delayed the egg is retained for longer than normal in the uterus and, consequently, can acquire characteristic eggshell abnormalities. These abnormal eggs are classified as either dusted or white-banded, the type of defect being related to the duration of oviposition delay (Hughes *et al.*, 1986). In the case of white banding the subsequent egg is also defective and is described as slab-sided. Examples of these three eggshell abnormalities are shown in Figure 1.2.

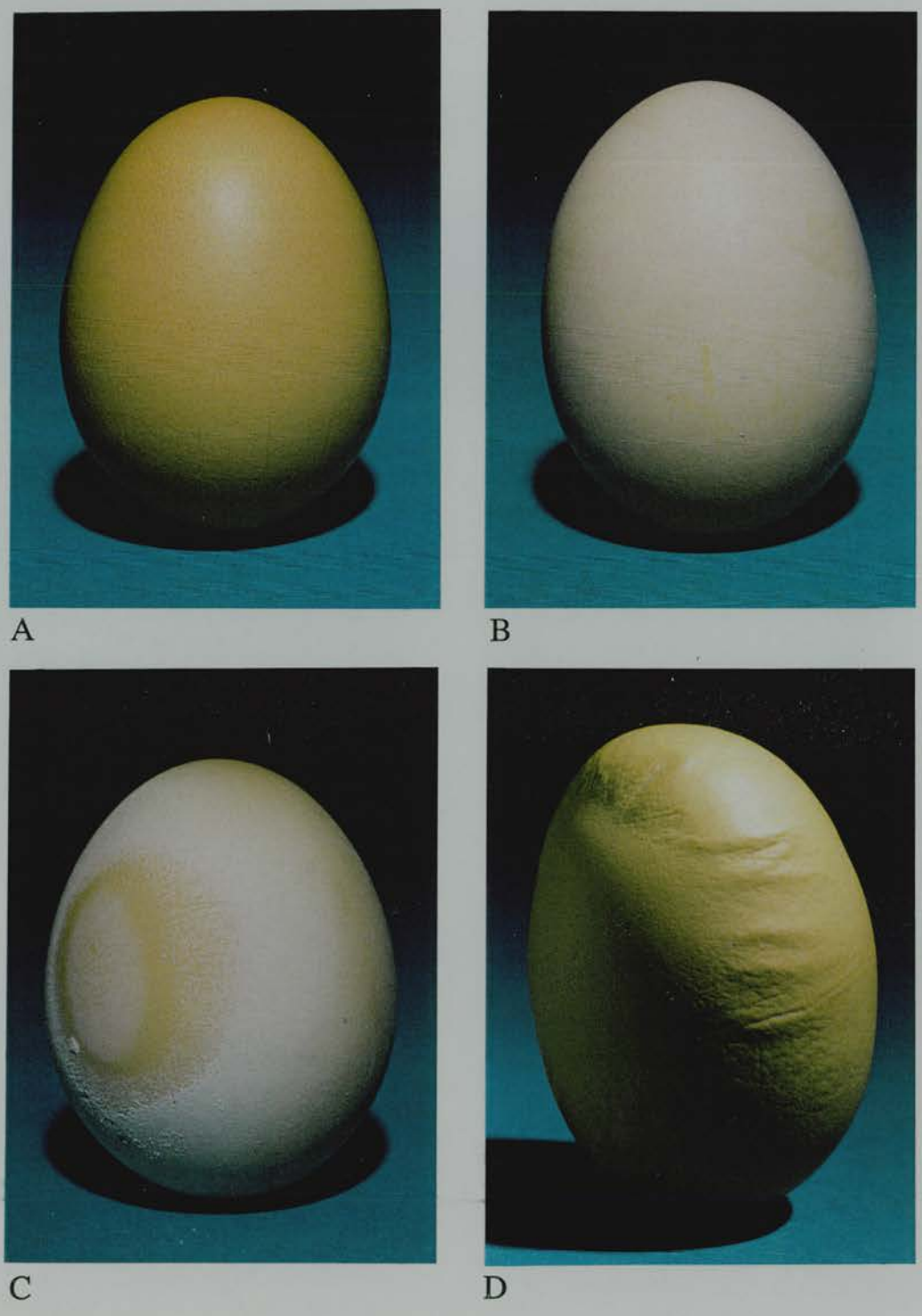



**Figure 1.2** Eggshell abnormalities associated with delayed oviposition:
A, normal egg; B, dusted egg;
C, white-banded egg; D, slab-sided egg.

Eggs laid after short oviposition delays tend to carry a coating of amorphous calcium carbonate on top of the cuticle (Hughes *et al.*, 1986). Although this additional layer is visible as a chalky white coating on the surface of brown eggs, and is described as eggshell dusting, it is difficult to detect on the white-shelled eggs produced by some strains of hen (Mauldin, 1992). Eggshell dusting can also occur in the absence of oviposition delays (Mills *et al.*, 1991).

When oviposition is delayed for longer periods, the next day's egg eventually arrives in the uterus and presses against the retained egg (Scott, 1940; van Middelkoop, 1971). At this stage the newly arrived egg does not yet have a shell and is therefore compressed where it touches the delayed egg. This deformation is fixed by calcification as the eggshell is formed. When the delayed egg is eventually laid it carries a prominent white band of additional calcification, indicating the area of contact between the two eggs, and is described as being white-banded. The subsequent egg is laid with a flattened area of eggshell and is described as slab-sided. In addition to their defective shape, slab-sided eggs tend to have thinner shells than normal eggs and may be laid prematurely without a shell (soft or shell-less eggs). White-banded eggs are sometimes referred to as A eggs and slab-sided eggs as B eggs, truncated eggs or compressed-side eggs (Romanoff & Romanoff, 1949; van Middelkoop, 1971; Carter, 1977). Hughes *et al.* (1986) observed that the mean duration of oviposition delay was 1.35 h for dusted eggs and at least 13 h for white-banded eggs.

Eggshell abnormalities similar to those resulting from oviposition delays can result from diseases such as infectious bronchitis (Spackman, 1987).

### 1.3.2 Downgrading of Abnormal Eggs

Eggs intended for domestic consumption are downgraded if they have eggshell abnormalities associated with oviposition delays and this results in a loss of revenue for the poultry industry. Consumers tend to discriminate against these eggs even though the contents are unaffected, and the slab-sided eggs are prone to cracking on account of their thinner shells. Although these eggs can be marketed through catering outlets or as liquid egg products, their value is much less than that of top grade eggs.

Eggs produced by breeding flocks for incubation are also downgraded if they have shell abnormalities associated with oviposition delays and this is because their hatchability can be reduced in two ways. First, the additional calcium carbonate deposits that form dusting and white-banding may block pores, thus reducing

eggshell porosity (van Middelkoop, 1971; Hughes *et al.*, 1986; Brake, 1987; Mauldin, 1992). Embryos developing in low porosity eggshells have reduced metabolic rates, reach low hatch weights and have a reduced chance of hatching relative to those in high porosity eggshells (Tullett & Deeming, 1982; Burton & Tullett, 1983; Peebles & Brake, 1987). Artificially reducing eggshell porosity by covering areas with tape restricts the exchange of the embryo's respiratory gases, thus leading to haematological and morphological changes which resemble those of ascitic birds (Maxwell *et al.*, 1987). Indeed, 88-98% of embryos incubated within white-banded eggs die during incubation (van Middelkoop, 1972). Second, eggs whose ovipositions have been delayed are kept at the hen's body temperature for longer than eggs laid normally and, consequently, may be at an over-advanced stage of development when laid. Patterson (1909) observed that eggs laid 20 h overdue contained embryos that were at a developmental stage equivalent to normally laid eggs that had been incubated for about 20 h. Storing eggs prior to incubation retards the development of the embryos, this effect being greatest for embryos laid at more advanced stages (Mather & Laughlin, 1979). Thus, by altering the stage of embryonic development at oviposition, oviposition delays may lower egg hatchability following storage.

From an animal welfare perspective, eggshell abnormalities associated with oviposition delays might provide a useful indicator of stress experienced by flocks of hens (Hughes *et al.*, 1986; Mills *et al.*, 1987b; Mills *et al.*, 1991). In addition to abnormalities resulting from oviposition delays, other eggshell defects indicate that hens have been exposed to stress and can sometimes be used to pinpoint the time of this disturbance (Coleman, 1988). For example equatorial bulges result from stress in the afternoon (Hughes & Black, 1976; Hughes *et al.*, 1986). A consideration of all stress-related eggshell defects, including those associated with oviposition delays, may be useful for assessing the welfare of commercial farmed hens.

### 1.3.3 Incidence of Abnormal Eggs

It is not clear what proportion of eggs laid by commercial flocks for consumption are downgraded due to eggshell abnormalities associated with oviposition delays. There are two reasons why this information is not available. First, egg producers routinely remove many eggs which carry major downgrading abnormalities before sending the better quality eggs to a packing station where further grading takes place. Although detailed breakdowns of the downgrading at grading stations is available, information on the downgrading carried out on farms is

not and so the proportion of all eggs laid with specific abnormalities is usually not known. The following surveys of downgrading considered all eggs laid. Second, published analyses of eggshell quality and downgrading group shell faults into categories which are not exclusively related to oviposition delays.

In a survey of more than 15 million eggs from 685 UK farms it was found that 7.06% were downgraded and that 90.54% of these downgradings (6.39% of all eggs) were due to shell faults (Maddison, 1970). Eggs with abnormalities associated with delayed oviposition will be included in the 6.39% of eggs downgraded due to shell faults. In a smaller but more detailed study, 500 eggs produced by ISA Brown hens in several farms were graded (see Table 1.1). Dusted and white-banded eggs would be classified as dusted or target eggs while slab-sided eggs would be included in the poor shell, mis-shapen, soft-shell and liquid categories. Thus, the proportion of eggs downgraded due to abnormalities associated with oviposition delays was less than 2.39%.

| Egg fault | | % of all eggs laid |
|---|---|---|
| Cracks | | 2.5 |
| Poor shell (e.g. thin shell) | * | 0.9 |
| Pin hole | | 0.7 |
| Mis-shapen | * | 0.9 |
| Soft-shell | * | 0.1 |
| Liquid | * | 0.13 |
| Dusting | * | 0.33 |
| Target (dusting on one pole) | * | 0.03 |
| White (lack of pigment) | | 0.2 |
| Internal egg faults | | 0.4 |
| Blood smears | | 0.2 |
| Hen soiled | | 4.0 |
| Yolk soiled | | 0.18 |
| All faults | | 10.57 |

**Table 1.1** The incidence of various categories of egg fault recorded in a study of 500 eggs from ISA Brown hens in several UK farms (information supplied in confidence by a commercial egg producing firm). Eggshell abnormalities associated with oviposition delays will contribute towards the categories marked '*'.

Carter (1977) found that the proportion of all eggs laid which were abnormal was 8.3, 9.2 and 0.5 %, respectively, for a medium body weight commercial brown-egg laying strain, a commercial White Leghorn strain and an experimental strain of Brown Leghorn. In that study, more than two-thirds of the recorded abnormal eggshells were white-banded or slab-sided. Ivy *et al.* (1972) reported that the incidence of abnormal eggs was 1.6% in a commercial flock of White Leghorn hens and 1-1.7% in two experimental strains of White Leghorn. These authors also noted that individual hens which laid white-banded and slab-sided eggs regularly could be identified, and suggested that removing a few of these birds would greatly reduce the overall incidence of these abnormalities in the flock. Jaap and Muir (1968) reported that 0.7-1.9% of eggs laid by two strains of Leghorn layer were abnormal and that a higher proportion, 1.5-5.9%, of eggs from broiler fowls were abnormal.

Both the incidence of dusted eggs and the incidence of white-banded and slab-sided eggs are heritable traits (Carter, 1977; Mills *et al.*, 1991). However, selection for production of these eggshells is limited by the low hatchability of embryos developing within them (van Middelkoop, 1972).

## 1.4 Physiology of Oviposition

### 1.4.1 Introduction

Oviposition involves vigorous contractions of the uterine muscles and relaxation of the uterovaginal sphincter, and is aided by the abdominal muscles (see Shimada and Saito (1989) for a comprehensive review). Prostaglandins (PGs) released into the circulation by ovarian follicles cause uterine muscles to contract more frequently. These contractions stimulate the posterior pituitary to release arginine vasotocin (AVT) into the circulation. AVT acts to further increase the frequency of uterine contractions, both directly and by inducing production of PGs by the uterine tissues themselves. The inter-relationships between PG and AVT release form a positive feedback loop which has a cascading effect on the development of uterine contractions. These contractions, aided by relaxation of the utero-vaginal sphincter, expel the egg from the oviduct. A diagrammatic representation of the mechanisms underlying oviposition is shown in Figure 1.3.

Uterine muscle contraction frequency varies during the laying cycle and this can be devided into three phases (Figure 1.4): 1) little activity while the egg is in the upper oviduct, 2) sustained medium frequency lasting 18-22 h coinciding with the

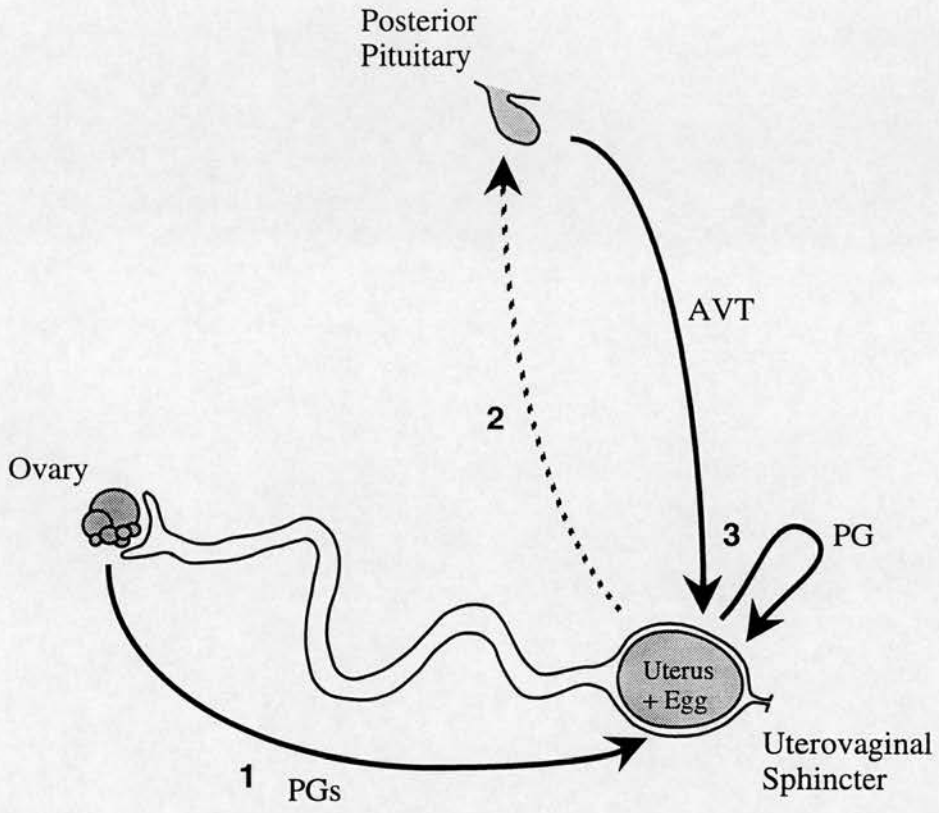



**Figure 1.3** Diagrammatic representation of mechanisms involved in the control of oviposition. 1) PGs, including PGF2α, released from the largest pre- and post-ovulatory follicles stimulate uterine contractions. 2) These contractions trigger AVT release from the posterior pituitary. 3) AVT further stimulates uterine contractions, both directly and by stimulating PG production in uterine tissues. These contractions, aided by PGE induced relaxation of the uterovaginal sphincter, lead to expulsion of the egg.

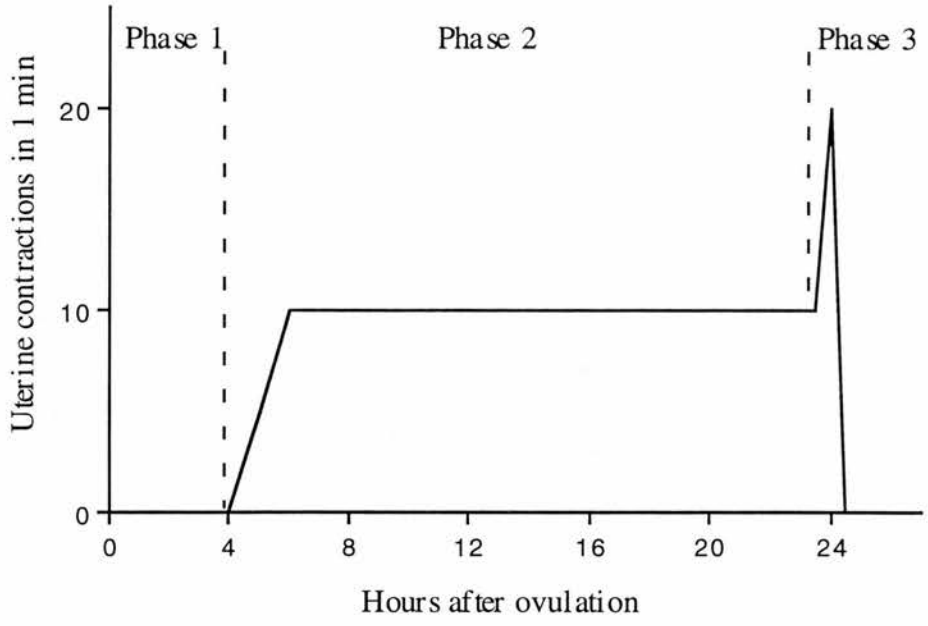



**Figure 1.4** Diagrammatic representation of the changes in uterine contraction frequency associated with the formation and oviposition of an egg, with oviposition occurring 24 h after ovulation (Shimada & Asai, 1978). See text (p. 10 & 12) for description of phases 1-3.

presence of an egg in the uterus and eggshell formation, 3) high frequency contractions at the time of oviposition (Shimada & Etches, 1986). These high frequency contractions which expel the egg from the uterus begin only 3-5 min before the egg is laid (Wechsung & Houvenaghel, 1988), and are likely to reflect the release of PGF2α and AVT (Shimada & Asai, 1978; Saito *et al.*, 1987). An increase in uterine contractions similar to phase 3 occurs about 30 min before the ovulation of first egg in a sequence, even though no egg is laid at that time (Shimada & Asai, 1978).

### 1.4.2 Prostaglandins

Plasma PG concentrations are elevated at the time of oviposition (Olson & Hertelendy, 1981) and it is thought that release of ovarian PGs, including PGF2α, is the initial event triggering a cascade of processes which lead to expulsion of the egg. Although there are some inconsistencies between studies, it appears that PGF2α accumulates in the largest pre- and post-ovulatory follicles of the ovary during the

hours before oviposition (Day & Nalbandov, 1977; Hammond *et al.*, 1980; Olson *et al.*, 1986; Saito *et al.*, 1987). Although the pre-ovulatory changes in plasma LH concentration are thought to influence oviposition time (Etches *et al.*, 1990), the mechanisms controlling ovarian PG release and the sequence of events linking the LH surge to the oviposition which occurs 4-6 h later are not yet understood.

Circulating PGF2α acts via specific receptors in the uterus to increase the contractile activity of the uterine muscles. The rise in plasma AVT concentration that is stimulated by these contractions (see below) further enhances the uterine contractions both directly and by stimulating the production of PGs, including PGE, within the uterine tissues (Rzasa, 1984; Goto *et al.*, 1985). In addition to promoting uterine contractions, PGE stimulates relaxation of the uterovaginal sphincter to facilitate the passage of the egg (Verma *et al.*, 1976; Wechsung & Houvenaghel, 1976).

Supporting the view that PG release is a cause rather than a consequence of oviposition, ovarian PG is not released in response to either intra-venous AVT injection or intra-uterine PG injection, both of which induce uterine contractions and premature oviposition (Olson & Hertelendy, 1981; Saito *et al.*, 1987). Furthermore, oviposition is induced by injection of PGs (Hertelendy *et al.*, 1974; Goto *et al.*, 1985) and delayed by injection of indomethacin, an inhibitor of PG synthesis (Shimada & Asai, 1979).

### 1.4.3 Arginine Vasotocin

AVT is a neurohormone synthesised in magnocellular neurones within the supraoptic and the paraventricular nuclei of the hypothalamus, and released from stores in the terminals of these nerves within the posterior pituitary (Goossens *et al.*, 1977). In addition to its role in oviposition, AVT is the avian anti-diuretic hormone and is physiologically important in the regulation of fluid balance (Stallone & Braun, 1986, Grossmann *et al.*, 1993). The physiological roles of mesotocin, an avian neurohypophysial hormone structurally similar to AVT and found in similar concentrations in the plasma, are largely unknown (Acher, 1993).

At the time of oviposition, the plasma AVT concentration rises to about 80 pg/ml and then rapidly declines, reaching basal levels of approximately 20 pg/ml after 15-30 min (Tanaka *et al.*, 1984). This rise in plasma AVT concentration is associated with a 30% depletion of the posterior pituitary store (Tanaka & Nakajo, 1962). Although AVT is also present in the ovary, it is unlikely that this source contributes to the rise in plasma AVT concentration at oviposition because the AVT

concentration in peripheral plasma is higher than that in follicular venous plasma at that time (Shimada *et al.*, 1987a). The rise in plasma AVT concentration promotes contractions of the uterus and thus assists with the expulsion of the egg. AVT acts via specific receptors on the uterus whose binding characteristics are altered in response to changes in PG and progesterone concentrations (Koike *et al.*, 1981; Takahashi *et al.*, 1992, 1994). Injection of exogenous AVT causes contractions of the uterine muscles and induces oviposition within a few minutes (Rzasa & Ewy, 1970).

Circulating AVT is rapidly degraded by an aminopeptidase, the plasma concentration of this enzyme being lowest around the time of oviposition and rising a few hours afterwards (Gilbert & Lake, 1964; Brzezinska *et al.*, 1967). The AVT inactivating activity of homogenates of various oviducal sections containing this enzyme was found to be greatest for the uterus (Brzezinska-Slebodzinska & Rzasa, 1973; Brzezinska-Slebodzinska *et al.*, 1979). There, it was also found to be lowest at the time of oviposition, and to rise in the subsequent 4 hours (Brzezinska-Slebodzinska *et al.*, 1979).

Several lines of evidence suggest that the stimulus for the rise in plasma AVT concentration at the time of oviposition is increased activity of uterine muscles. First, plasma AVT concentration does not rise at normal oviposition time when the oviposition is delayed by indomethacin, an inhibitor of PG synthesis, suggesting that AVT release is a response to either elevated PG concentration or increased uterine contractions. Second, when oviposition is induced prematurely by intra-uterine injection of PG, acetylcholine or orthophosphate solution, plasma AVT concentration increases but plasma PG concentration does not (Saito *et al.*, 1987a; Shimada *et al.*, 1987; Murakami *et al.*, 1991). Third, distension of the oviduct by either digital palpation or inflation of an inserted balloon stimulates AVT release, transiently doubling basal plasma levels (Rzasa *et al.*, 1979; Shimada *et al.*, 1987). Although the mechanism by which uterine contractions stimulate AVT release has not been described, a neural pathway similar to that which triggers oxytocin release during parturition and lactation (Edqvist & Stabenfeldt, 1993) may be involved.

It is still unclear whether AVT is essential for oviposition, as hens which have been neurohypophysectomised (surgical removal of the posterior pituitary) are reported to lay normally even when this procedure is carried out only 1-2 hours before oviposition, thus ruling out any possibility of neural regrowth (Shirley & Nalbandov, 1956; Opel, 1965; Nakada *et al.*, 1993, 1994). Furthermore, hens with electrical lesions in the supraoptic nucleus show polydipsia, suggesting little anti-diuretic release of AVT, yet resume laying a few days after surgery (Ralph, 1960).

However, in all these experiments, the possibility that AVT was still released in association with oviposition, e.g. from cut nerve endings, cannot be ruled out. As yet, no experiment measuring plasma AVT concentrations associated with ovipositions of neurohypophysectomised hens has been reported. Nakada *et al.* (1994) reported that injection of AVT antiserum, at a dose considered sufficient to neutralise all circulating AVT, did not cause oviposition delays, further suggesting that AVT may not be essential for oviposition. However, no measurements of circulating AVT concentrations associated with oviposition, needed to confirm the hypothesis, were made in that experiment.

An alternative approach to assessing the importance of AVT in the oviposition process would be to use AVT analogues which block AVT receptors, thus preventing any action of AVT. A similar approach using an appropriate analogue has been used to demonstrate that oxytocin is essential for the normal progression of parturition in rats (Antonijevic *et al.*, 1995). Currently, the most potent AVT analogues are able to block AVT induced contractions of isolated strips of uterine muscle for only a few minutes, and so this approach is not yet able to assess the importance of AVT in oviposition (Sudo *et al.*, 1988).

## 1.5 Possible Mechanisms Underlying Stress-Induced Oviposition Delays

As described in the previous section, uterine contractions which expel the egg at oviposition are initiated by ovarian PGs and then promoted further by neurohypophysial AVT. Four ways in which stress may act to delay oviposition are now hypothesised.

First, stress might delay the initiation of oviposition by preventing ovarian PG release. The fact that either removal of the largest pre- or post-ovulatory follicle, or treatment with a prostaglandin inhibitor delays oviposition (Rothchild & Fraps, 1944; Gilbert *et al.*, 1982; Hertelendy & Biellier, 1978) suggests that ovarian PGs are indeed essential for oviposition. Hence, any stress-related inhibition of PG release (direct or indirect) could presumably delay oviposition.

Second, stress might inhibit neurohypophysial AVT release and so interrupt the cascade of processes which cause oviposition. In mammals release of oxytocin, a neurohypophysial hormone with functions similar to those of AVT, is inhibited by endogenous opioids released in response to stress, resulting in interruptions to parturition and lactation (see Petherick *et al.* (1993) for a review). By analogy, a stress-related opioidergic inhibition of AVT release may be the cause of oviposition

delays. The literature relating to this hypothesis is reviewed in Section 1.6.

Third, stress may directly suppress development of the uterine contractions required to expel the egg. Adrenaline is released in response to various stressors (Freeman, 1976; Harvey *et al.*, 1984), and exogenous adrenaline relaxes uterine muscles, both *in vitro* and *in vivo,* (e.g. Crossley *et al.,* 1980; Wechsung & Houvenaghel, 1987) and delays oviposition (e.g. Crossley, 1983). Hence, oviposition delays could be due to stress-related adrenergic suppression of uterine contractions. The literature relating to this hypothesis is reviewed in Section 1.7.

Fourth, stress may cause contractions of the utero-vaginal sphincter and so prevent uterine contractions from expelling the egg. The sphincter is known to posses musculature appropriate for this (Gilbert & Lake, 1963).

## 1.6 Opioids and Stress: Their Effect on Neurohypophysial Hormone Release

In this section the effects of opioids on neurohypophysial hormone release and their possible role in mediating oviposition delays during stress are reviewed. From this evidence, it is hypothesised that endogenous opioids released in response to stress may induce oviposition delays by inhibiting neurohypophysial AVT release.

### 1.6.1 Opioids and Opioid Receptors

Opioid peptides are found in many organs where they are involved in various physiological processes including responses to physical and psychological stress (see Akil *et al.* (1984) and Millan and Herz (1985) for reviews on opioid biology). There are three families of endogenous opioids: endorphins, enkephalins and dynorphins. Within each family, opioids are derived in specific neurones by cleavage of one precursor polypeptide.

From behavioural studies, radioligand binding assays and *in vitro* pharmacological bio-assays, three major types of opioid receptor have been identified (Mansour *et al.*, 1988, Simon & Gioannini, 1993). Each of these receptor types can be further classified into sub-types. These receptors show preferential binding for particular opioids and have different anatomical distributions and proposed physiological roles (Akil *et al.*, 1988). When stimulated, opioid receptors interact with G-proteins and so influence cell function by altering the permeability of ion channels or by causing production of intracellular second messengers such as cAMP (see North (1993) and Childers (1993) for reviews).

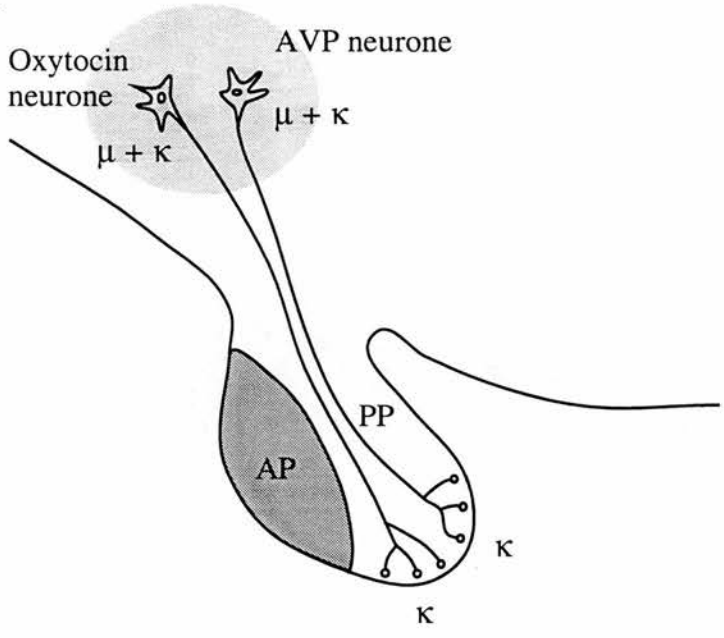
For mammals, DNA cloning and expression techniques have been used to describe the structure of each opioid receptor type and its interactions with ligands (Knapp *et al.*, 1995). Although avian opioid receptors are assumed to be similar to those of mammals, their molecular biology has not yet been described by cloning and the possibility that structural differences could cause them to interact differently with ligands cannot be excluded. However, studies into the distribution of opioid receptor types have found that ligands specific for mammalian mu, kappa and delta sites have similar binding characteristics and selectivity in avian tissues, suggesting that there is structural similarity between mammalian and avian receptors (Reiner *et al.*, 1989; Csillag *et al.*, 1990; Deviche *et al.*, 1993).

Various agonists and antagonists which respectively stimulate and block opioid receptors are available and can be used to manipulate opioid pathways experimentally (Zimmerman & Leander, 1990; McKnight & Rees, 1991). The selectivity of these compounds for specific types of opioid receptor is assessed from binding assays, a technique which determines their abilities to displace specific radio-labelled ligands from each of the receptor types, usually in rat brain

| | Relative affinity for specific receptor type | | | |
|---|---|---|---|---|
| | mu | delta | kappa | Reference |
| Antagonists | | | | |
| Nalmefene | 1 | 0.16 | 0.19 | Michel *et al.*, (1985) |
| MR2266 | 0.7 | 0.12 | 1 | Magnan *et al.* (1982) |
| norBNI | 0.02 | 0.01 | 1 | Traynor *et al.* (1989) |
| Agonists | | | | |
| Morphine | 1 | 0.03 | <0.01 | Corbett *et al.* (1993) |
| U50,488 | <0.01 | <0.01 | 1 | Corbett *et al.* (1993) |

**Table 1.2** Relative abilities of three antagonists and two agonists to displace specific ligands from each of the three opioid receptor types. For each compound, the receptor type at which the ligand was most readily displaced has a relative affinity of 1. Binding affinity information was calculated from data in the references provided. Note that different ligands were used in studies reported by different authors.

**Mammalian**




**Avian**

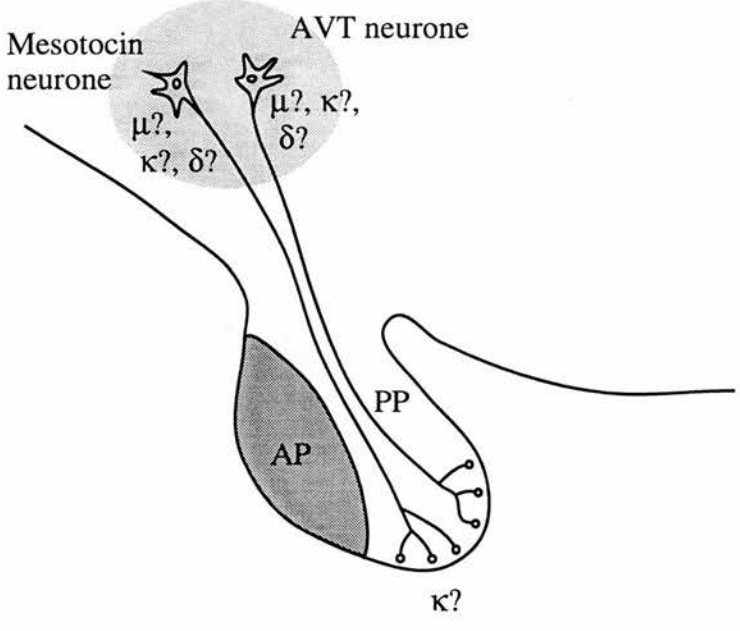



**Figure 1.5** Diagrammatic representation of the mammalian and avian magnocellular neurones showing the distribution of specific opioid receptors which may influence hormone release. Note that the hypothalamus (indicated by light shading), but not the pituitary, is contained within the blood brain barrier. Where information on the distribution of opioid receptors in hens is not available this is indicated by a '?'. The literature relating to this figure is reviewed in the text (p. 19 & 20). PP, posterior pituitary; AP, anterior pituitary; μ, mu receptor; κ, kappa receptor; δ, delta receptor.

preparations (Corbett *et al.*, 1993). The agonistic or antagonistic nature of these compounds is usually identified from their effects in peripheral tissue bioassays (Corbett *et al.*, 1993; Smith & Leslie, 1993). Table 1.2 shows the opioid receptor selectivities of three antagonists and two agonists used in this project.

### 1.6.2 Neurohypophysial Hormones and Opioids

In mammals, the neurohypophysial peptides oxytocin and vasopressin (AVP) have evolved from mesotocin and AVT respectively, and functionally assume both of the physiological roles of AVT (Archer, 1993). Oxytocin promotes milk ejection in response to suckling and uterine contractions during parturition, while AVP, the mammalian anti-diuretic hormone, is important in the regulation of water balance and in the maintainence of cardiovascular function during haemorrhage. In addition to its osmoregulatory functions, AVP has a role in controlling physiological responses to stressors - in particular it facilitates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary (Krishnan *et al.*, 1991). In hens, AVT is released in response to challenges where plasma osmolality is not affected, such as hatching (Kisliuk & Grossmann, 1994), and its role in the physiological stress response may be similar to that of AVP in mammals (Kuenzel & Blahser, 1989).

These peptides are synthesised in separate groups of magnocellular neurones within the paraventricular and supraoptic nuclei of the hypothalamus, and are passed down the axons arising from these cells to be stored in their nerve terminals in the neurohypophysis (posterior pituitary). Nerve impulses travelling down this hypothalamo-hypophysial pathway trigger release, by exocytocis, of oxytocin and AVP from these stores into the circulation.

Opioids can block the secretion of oxytocin and AVP both by acting to reduce the electrical discharge activity of the magnocellular cell bodies in the hypothalamus (centrally) and by inhibiting the exocytosis of storage vesicles in the nerve terminals in the neurohypophysis (peripherally) (see Bicknell (1985) for a review). Opioid agonists and antagonists which alter the release of neurohypophysial hormones may only be assumed to act peripherally if it can be shown that the firing rate of the magnocellular neurones is unchanged.

The opioidergic control of magnocellular neurones has been investigated by describing the distributions of specific opioid receptor types and by characterising the effects of specific opioid agonists and antagonists on peptide release. In mammals both mu and kappa receptors in the hypothalamus modulate neurohypophysial peptide release, while in the pituitary only kappa receptors are

involved (Bicknell, 1985, 1993). In birds, opioidergic control of the neurohypophysial system has not been fully described. Although the avian paraventricular nucleus contains mu, kappa and delta receptors, it is not known if these are associated with the cell bodies of the magnocellular neurones (Deviche *et al.*, 1993). Kawashima *et al.* (1995) found that the neurohypophysis of the laying hen contained a single type of opioid receptor (type not identified) and suggested that it may be involved in regulating peptide release. Martin *et al.* (1992) found that specific opioid binding sites in the hen were associated with the terminals of mesotocin neurones but not with those of AVT neurones. Presumably these binding sites were the single opioid receptor type identified by Kawashima *et al.* (1995) and, by analogy with the mammalian neurohypophysial system, it seems likely that they will be kappa type.

Figure 1.5 shows diagrammatic representations of mammalian and avian neurohypophyses and indicates the distribution of specific opioid receptors which may influence hormone release.

### 1.6.3 Opioid Modulation of Oxytocin and Vasopressin Release

Centrally, endogenous opioids act via both mu and kappa receptors to modulate electrical activity in the hypothalamo-neurohypophysial pathway and hence oxytocin release from the neurohypophysis. Both morphine and U50,488, selective mu and kappa opioid receptor agonists respectively, reduce the spontaneous firing rate of putative oxytocin neurones in the rat hypothalamus both *in vitro* (Wakerley *et al.*, 1983; Pumford *et al.*, 1992) and *in vivo*, following intravenous administration, (Pumford *et al.*, 1991, 1992, 1993).

Peripheral opioidergic inhibition of oxytocin release is mediated by kappa receptors, with U50,488 inhibiting oxytocin secretion from the electrically stimulated rat neurohypophysis *in vitro* (Zhao *et al.*, 1988a) and *in vivo* (Russell, 1993). Mu receptor agonists such as morphine do not inhibit the release of oxytocin caused by electrical stimulation of the neural stalk (Russell *et al.*, 1993). Endogenous opioids can also inhibit the release of neurohypophysial noradrenaline and thus, as noradrenaline facilitates oxytocin secretion from the nerve terminals, can suppress oxytocin release indirectly at the level of the neurohypophysis (Dyer 1988, Zhao *et al.* 1988b).

Injections of morphine or U50,488 depress circulating oxytocin concentration, causing prolonged labour in rats. This disruption of parturition can be ended by intravenous infusions of exogenous oxytocin or prevented by treatment

with naloxone (Gosden *et al.*, 1985; Russell *et al.*, 1989; Douglas *et al.*, 1993). Similarly, morphine blocks suckling induced milk ejection in rats and mice in a naloxone reversible manner (Haldar & Sawyer, 1978; Russell & Spears, 1983). During normal parturition, modulation of oxytocin release by endogenous opioids may play a role in spacing the deliveries of successive births (Leng *et al.*, 1985).

AVP and oxytocin neurones themselves also release opioids which may be involved in regulation of neurohypophysial hormone release (van Wimersma Greidanus & van de Heijning, 1993). For example, dynorphin is co-released with oxytocin and can act via peripheral kappa opioid receptors to inhibit oxytocin release (Leng *et al.*, 1994).

### 1.6.4 Opioids, Oxytocin and Stress-Induced Disruption of Parturition and Lactation

Disturbing rats or pigs in mid-parturition by moving them to an unfamiliar environment increases the inter-birth interval, thus prolonging labour. This disruption is reduced by the non-selective opioid receptor antagonist naloxone, thus indicating a role of opioids in the mechanisms underlying this response (Leng *et al.*, 1987; Leng *et al.*, 1988; Lawrence *et al.*, 1992). The drop in plasma oxytocin concentration which is associated with the stress-induced increase in inter-birth interval does not occur in naloxone treated animals. Furthermore, administration of exogenous oxytocin to rats or pigs that are exposed to disturbance causes labour to resume. These observations indicate that stress prolongs parturition in mammals by inhibiting oxytocin release in an opioidergic manner. Similarly, stress-related inhibition of suckling-induced milk ejection in mice is reversed by oxytocin or naltrexone treatments, again suggesting that opioidergic inhibition of oxytocin release is involved (Haldar & Bade, 1981).

### 1.6.5 Opioids and Arginine Vasotocin Release

In both day-old chicks and laying hens, the rise in plasma AVT concentration associated with elevated plasma osmolality following hypertonic saline injection is completely prevented by morphine and is enhanced by naloxone (Xu *et al.*, 1991; Dublecz *et al.*, 1992). It has also been reported that the peak in plasma AVT concentration accompanying oviposition is increased almost two-fold by treatment with naloxone (F.Ellendorff, personal communication).

Given that stress inhibits oxytocin release via opioidergic pathways in mammals, leading to disrupted parturition and milk ejection, and that opioidergic

pathways in the hen can inhibit AVT release in response to osmoregulatory stimuli, it is hypothesised that stress-induced oviposition delays could be due to opioidergic inhibition of neurohypophysial AVT release.

### 1.7 Influence of Adrenaline on Uterine Contractions

In the oviduct of hens there are both alpha and beta adrenergic receptors, the concentrations of which vary between different oviduct segments (Verma & Walker, 1974; Crossley *et al.*, 1980). Adrenergic stimulation of alpha receptors induces contraction in the smooth muscle of the oviduct while stimulation of beta receptors causes relaxation. In the uterus the adrenergic receptors are mostly beta while those in the uterovaginal sphincter are mostly alpha. As a result of this uneven distribution, adrenergic stimulation causes relaxation of uterine muscle and contraction of uterovaginal sphincter muscle, both *in vitro* and *in vivo* (Sykes, 1955; Chen & Hawes, 1967; Verma *et al.*, 1977; Crossley *et al.*, 1980; Wechsung & Houvenaghel, 1987).

Oviposition delays can be induced by injecting hens with adrenaline (Sykes, 1955; Crossley, 1980; Hughes & Gilbert, 1984). Indeed, injections of pituitrin, a posterior pituitary extract containing AVT, are unable to induce oviposition in hens that have been injected with adrenaline (Sykes, 1955). The induction of oviposition delays by exogenous adrenaline is prevented by including propranolol, a beta adrenergic receptor blocker, in the hens' diet (Crossley, 1983).

The release of adrenaline from the adrenal medulla into the circulation is involved in mediating an animal's immediate responses to stress, and this has been demonstrated for birds (Freeman, 1976; Harvey *et al.*, 1984). It has been suggested that adrenaline released in response to stress might cause oviposition delays by directly inhibiting uterine contractions (Weiss & Sturke, 1952; Sykes, 1955; Hughes & Black, 1976; Crossley, 1983). Crossley (1983) reported that saline injections caused oviposition delays of about 4.5 h duration and that these were prevented by dietary propranolol. It was suggested that the saline injections were stressful and induced oviposition delays by causing the release of endogenous adrenaline which suppressed uterine contractions. It is not known if propranolol can reduce or prevent induction of oviposition delays by environmental stress.

Bostedt and Rudloff (1983) reported that carazolol, another adrenergic beta-blocker, reduced the duration of parturition in sows and suggested that the stress of labour itself causes adrenaline release and a consequent suppression of uterine

contractions. Prolonged parturition in women may be due partly to increased levels of circulating adrenaline associated with stress (Lederman *et al.*, 1985). Inhibitory adrenergic pathways have also been implicated in mechanisms underlying stress-induced disruption of parturition in mammals (Bontekoe *et al.*, 1977; Bosc & Nicolle, 1979).

Stress-related beta adrenergic pathways inhibit induction of uterine contractions (and hence oviposition) by AVT in the lizard *Anolis carolinensis*, and by PGF2α in the gecko *Haplodoctylus maculatus* (Jones *et al.*, 1983; Summers *et al.*, 1985; Cree & Guillette, 1991). However, at this time the uteri of *A. carolinensis* and *H. maculatus* still respond to PGF2α and AVT, respectively, and so it is likely that adrenaline is inhibiting the response to specific hormones rather than the ability of the uterus to contract.

## 1.8 Outline of Thesis

The aim of this thesis is to describe the induction of oviposition delays by environmental stress and to investigate the physiological mechanisms underlying these delays. Several hypotheses accounting for these delays are proposed in Section 1.5. The experiments reported here investigated two of these hypotheses in particular: firstly, opioid peptides released in response to stress may inhibit AVT release; secondly, adrenaline released in response to stress may directly suppress uterine contractions.

Procedures common to several experiments are described in Chapter 2.

The aim of Chapter 3 is to describe the duration of oviposition delays caused by social stress (relocation of hens from individual to group cages) and how this is influenced by the timing of the stress. The suitability of social stress as a model for investigating the physiological causation of these delays is confirmed. Consequences of these oviposition delays for eggshell quality are also examined.

The hypothesis that stress delays oviposition by inhibiting neurohypophysial AVT release predicts that plasma AVT concentrations will rise in association with delayed oviposition but not during the delay. Furthermore, it predicts that plasma PG concentrations are elevated both during the delay (as PG induced uterine contractions are the stimulus for AVT release) and also in association with the delayed oviposition. An alternative hypothesis, that delays are caused by stress-related inhibition of ovarian PG release, also makes predictions concerning changes in plasma PG and AVT concentrations associated with oviposition delays. In order

to examine these possibilities, the aim of Chapter 4 is to examine changes in plasma PG and AVT concentrations associated with stress-induced oviposition delays.

The hypothesis that stress delays oviposition by inhibiting AVT release predicts that elevated uterine contraction frequency, the stimulus for AVT release, occurs during these delays. The aim of Chapter 5 is to examine uterine contraction frequencies associated with stress-induced oviposition delays.

If stress-induced oviposition delays are caused by a mechanism other than inhibition of ovarian PG release, then these same processes might impair the ability of exogenous PG injections to induce oviposition prematurely. The first aim of Chapter 6 is to examine the effect of stress on the induction of premature oviposition by exogenous PGF2$\alpha$. If oviposition delays are due to a stress-related inhibition of hormone release (either AVT or PGs) then injections of this hormone (either AVT or PGs, respectively) should elevate circulating concentrations and end delays by expelling the eggs. The second aim of Chapter 6 is to see if exogenous AVT or PGF2$\alpha$ would end stress-induced oviposition delays.

If stress-induced oviposition delays are mediated by endogenous opioids then their duration should be reduced by blocking the appropriate opioid receptors with an antagonist. The aim of Chapter 7 is to investigate the effects of opioid receptor antagonists on the duration of oviposition delays.

If endogenous opioids cause oviposition delays then exogenous opioids should also do so. Chapter 8 investigates the effects of two exogenous opioids, morphine and U50,488, on oviposition time.

It is hypothesised that opioids may delay oviposition by inhibiting AVT release. The aim of Chapter 9 is to investigate the effects of U50,488 on premature oviposition induction and associated AVT release caused by PGF2$\alpha$ injection.

In mammals a variety of stressors cause the release of endogenous opioids (Rodgers & Randall, 1988) and it is assumed that this is also the case in hens. The aim of Chapter 10 is to investigate the effect of social stress on opioid release using frequency of headflicking in response to alerting stimuli, a putative index of hens' opioid status, as a behavioural indicator.

It is hypothesised that adrenaline may act via beta adrenergic receptors to suppress uterine contractions, and hence to delay oviposition, during stress. The aim of Chapter 11 is to test this hypothesis through treatments with propranolol, a beta adrenergic receptor blocker.

Chapter 12 provides a general discussion of stress-induced oviposition delays and their underlying causation.

The method used to calculate when hens would normally have laid for days

on which there was an experimental manipulation is validated in Appendix 1. Validation of the radioimmunoassay used to measure AVT in avian plasma is reported in Appendix 2. An investigation into the possibility that ovulation is also delayed by social stress is reported in Appendix 3.

# CHAPTER 2: **General Materials and Methods**

In this chapter the procedures common to most of the experiments in this thesis are described. Alternatives or modifications to these are described in the appropriate chapters.

## 2.1 Animals and Husbandry

Hens (*Gallus gallus domesticus*) of two commercial egg-laying strains, ISA Brown and Hisex, were used in this study. Except where Hisex is specified, experiments used ISA Brown hens. These birds were reared in groups in commercial brooders from day-old till 4 weeks of age, and then in groups in rearing cages for a further 12 weeks. At 16 weeks of age the hens were moved to the middle tier of a battery unit where they were housed individually in alternate cages (0.29 m wide x 0.46 m deep x 0.43-0.51 m high), so that none had immediate neighbours. The lighting regime was 14L:10D, and lights on time varied from 7.00-9.00 between experiments so that the peak period of oviposition occurred at a time convenient for the required observations or manipulations. Food (standard layer mash; 160 g/kg protein, 11.0 MJ/kg metabolisable energy, 33 g/kg calcium) and water were provided *ad libitum*. As most eggs were laid in the morning, husbandry procedures such as feeding and cleaning were done in the afternoon to avoid any influence on morning oviposition times.

Individual birds were identified by numbered wing bands or coloured leg rings. Occasionally, when experimental procedures involved housing birds in groups, individuals were numbered on their combs with a marker pen to facilitate identification without further handling.

For experiments on oviposition, only hens aged from 24 to 57 weeks were used. Before this many birds are not yet in regular lay. Towards the end of this period the lag increases, egg sequence length decreases and the frequency of missing eggs (e.g. those internally ovulated) increases (Lillpers & Wilhelmson, 1993a). As this makes it more difficult to predict oviposition times and accurately calculate expected oviposition times, younger birds were used where possible.

Mean body weights were 1.87 kg at 25 weeks of age (range: 1.7 to 2.1 kg) and 2.16 kg at 47 weeks of age (range: 1.7 to 2.6 kg).

## 2.2 Recording Oviposition Times

To start with each cage was fitted with automatic oviposition recording equipment, comprising a flap at the lower end of the sloping floor linked via a magnetic switch to a digital stop-clock. When an egg rolled down the floor the flap was deflected thus starting the stop-clock. Later, when the eggs were collected, the stop-clock times were deducted from the present time to give the time when the egg rolled out of the cage, and thus an indication of oviposition time. However, it became apparent that some eggs were not rolling from the cage immediately after oviposition and occasionally remained in the cage for several hours. Subsequently, cages were inspected visually every 10-20 min during peak laying hours to allow more accurate records of oviposition time to be made.

## 2.3 Definition and Calculation of Oviposition Times (Observed, Predicted and Expected), Oviposition Delay and Change in Oviposition Time

*Observed Oviposition Time*

The time when an egg was actually laid is described as the 'observed oviposition time'. The observed oviposition time is also referred to as the oviposition time.

*Predicted Oviposition Time*

From records of a bird's previous oviposition times it was often possible to estimate the time of the next oviposition. This estimate is described as the 'predicted oviposition time' and was used to determine when experimental procedures prior to oviposition should be carried out.

*Expected Oviposition Time*

As many of the experiments were designed to examine the effects of different treatments on oviposition time, it was necessary to calculate the 'expected oviposition times', i.e. the times when ovipositions would actually have occurred in the absence of any manipulation. This was done using the records of observed oviposition times on the days before and after the treatment. In the middle of long egg sequences the daily lag remains relatively constant, and for the calculations it was assumed that oviposition time advanced by a similar increment each day. Expected oviposition time was calculated as the mid-point between the observed oviposition times on the day

before and the day after treatment. The observed oviposition time for the day before treatment was always known (an essential prerequisite for treatment to proceed), but sometimes observed oviposition time was not available for the day after treatment, e.g. if this egg was laid with a soft shell and was therefore clearly premature. When this occurred, expected oviposition time was calculated using the observed oviposition time on the second day after treatment, again assuming that the lag was similar each day. The assumption that oviposition time advances by a similar amount each day is not valid for the first and last few eggs in a sequence (see reviews by Johnson (1986) and Etches (1990)), and thus expected oviposition times could not be calculated for these days. Consequently, where treatments occurred in either the first three or the last three eggs in a sequence, these data were excluded from the analyses.

Appendix 1 describes an experiment to assess the accuracy of this method of calculating expected oviposition time. When the expected oviposition times for 25 ovipositions were calculated using the observed oviposition times on the day before and on the day after, they were on average 0 min later than the observed oviposition times (95% confidence limits: -29 to 28 min). When expected oviposition times for the 25 ovipositions were calculated using the observed oviposition times on the day before and on the second day after, they were on average -2 min later than the observed oviposition times (95% confidence limits: -32 to 27 min).

*Oviposition Delay and Change in Oviposition Time (Δ Oviposition Time)*

The terms oviposition delay and Δ oviposition time describe how late an oviposition is with respect to the expected oviposition time.

The oviposition delay was calculated by deducting the expected oviposition time from the observed oviposition time. Thus, when oviposition occurs later than expected the oviposition delay is positive and when oviposition occurs earlier than expected the oviposition delay is negative. Following the findings in Chapter 3, oviposition delays longer than three hours were given a score of 180 min for the analyses. Where it could not be assumed that oviposition delays would be caused by some of the experimental treatments, the difference between observed and expected oviposition times is described as the change in oviposition time (Δ oviposition time). Thus, where oviposition occurs earlier than expected, Δ oviposition time is negative.

## 2.4 Experimental Design

From a stock of hens whose oviposition times were recorded, an appropriate number of birds whose next oviposition time could be predicted were selected and then allocated randomly to the treatments. Before treatment, the presence of a hard-shelled egg in the uterus of each selected hen was confirmed by palpation, a procedure which does not influence oviposition time (Juhasz & van Tienhoven, 1964; Hughes *et al.*, 1986).

Where possible, all birds in an experiment were treated on the same day and this should be assumed unless otherwise stated. However, because it was often not possible to confidently predict oviposition times for a sufficient number of birds on one day and some experimental protocols were too time-consuming to treat all birds on one day, some experiments took place over several days. On each day, selected birds were divided evenly and randomly between the treatments. Where appropriate, the potential for day effects was included in the statistical analysis.

In most experiments, treatments such as injections took place at a specific time before predicted oviposition time. However, as predicted oviposition time is only an estimate of expected oviposition time, inevitably there was a range of times (instead of a fixed time) between the treatment and the expected oviposition time.

## 2.5 Social Stress

In this thesis, relocation from individual cages to group cages was used as a standard stressor for the induction of oviposition delays. Several birds, normally four, were moved from their home cages (described in Section 2.1) to a group cage (1.16 m wide x 0.46 m deep x 0.50-0.58 m high) in the top tier of a battery. This procedure, involving both relocation and contact with unfamiliar birds, is referred to as social stress and is similar to that described by Watt (1989). As a result of the findings reported in Chapter 3, periods of stress did not normally extended for more than 3.5 h after the predicted oviposition time.

## 2.6 Injection and Blood Sampling Procedures

To receive intra-venous (i.v.) injections, birds were restrained on their sides and the brachial vein (wing vein) exposed by removing a few feathers. Solutions were injected with the syringe needle (25G) pointing towards the body then, once the

needle was withdrawn, temporary pressure was applied to the vein to prevent haematoma. Intra-muscular (i.m.) injections were via the pectoral muscle (breast muscle) with the bird restrained on its back. Injection volumes were proportional to body weight.

To obtain a blood sample, the wing vein was exposed as above and 1-2 ml blood was withdrawn with the syringe needle (25G) pointing away from the body. Temporary pressure was applied to the vein to prevent haematoma. Blood samples were transferred immediately to either micro-centrifuge tubes or sample tubes containing an appropriate anticoagulant, and chilled on ice. For the measurement of PGFM, 1.35 ml blood samples were placed in 1.5 ml micro-centrifuge tubes containing 15 µl of 100 mM aspirin (prostaglandin synthesis inhibitor) and 150 µl of 3.8% citrate in 0.9% saline (anticoagulant). For AVT measurement, heparin coated sample tubes were used. Following centrifugation at 1600 **g** for 10 min, the supernatant (plasma) was removed and stored at -18$^{o}$C until required.

## 2.7 Statistical Analysis

Many of the data obtained in this study were not distributed normally and were therefore analysed using nonparametric statistical tests, such as the Mann-Whitney test or sign test (Siegel & Castellan, 1988). When a more complex analysis was required, e.g. to allow for potential day effects, the data were transformed to produce a more normal distribution with an even distribution of variance. Parametric tests included the t-test, analysis of variance (ANOVA) and the General Linear Model (GLM, a form of analysis of variance which is robust for unbalanced data). When comparing means with similar variances by t-test, the pooled version of this test was used as this is slightly more powerful than the standard version. All statistical tests were two-tailed.

# CHAPTER 3: **Experiments on the Duration of Stress-Induced Oviposition Delays and Related Eggshell Abnormalities**

## 3.1 Introduction

Stressors, such as exposure to unfamiliar hens or relocation to novel environments, can cause hens to delay oviposition (Scott, 1940; Hughes, 1979; Hughes *et al.*, 1986; Watt & Solomon, 1988; Watt, 1989). For hens which regularly lay in nest sites, preventing or hindering access to these sites can also induce oviposition delays (Duncan, 1970; Kite, 1985; Hughes *et al.*, 1986; Cooper & Appleby, 1994). When oviposition is delayed for short periods, the egg typically acquires an additional layer of calcification which overlies the normal eggshell and is described as "dusting" (Hughes *et al.*, 1986). When oviposition is delayed for longer periods, the next egg eventually arrives in the uterus and presses against the delayed egg. As a consequence of this contact, the delayed egg's shell acquires a "white band" and the second egg's shell is formed with a flattened ("slab") side (Scott, 1940; van Middelkoop, 1971). Hughes *et al.* (1986) observed that the mean duration of oviposition delay was 1.35 h for dusted eggs and at least 13 h for white-banded eggs. These abnormalities reduce shell porosity of eggs intended for incubation, thus compromising the embryos' health, (van Middelkoop, 1972), and make eggs intended for domestic consumption unattractive to consumers (Hughes *et al.*, 1986). Consequently, these abnormal eggs are downgraded, resulting in loss of revenue for egg producers. From an animal welfare perspective, eggshell abnormalities associated with stress-induced oviposition delays might provide a convenient means of identifying stressful conditions to which hens have been exposed (Hughes *et al.*, 1986; Mills *et al.*, 1987b, 1991).

Although little is known about how the duration of oviposition delay is affected by the timing of exposure to environmental stress, all stressors reported to cause oviposition delays were present at the time when oviposition would normally be expected. Kite (1985) reported that hens which normally laid in nest boxes would regularly delay oviposition, for durations often exceeding 24 h, if access to the boxes was prevented. These hens would lay delayed eggs within a few minutes of nest boxes being made accessible again. In pilot experiments for the present study, transport simulation (horizontal vibration at 1 Hz) and relocation from single cages to group pens caused oviposition delays. Delayed eggs were usually laid within a few hours of expected oviposition time or not until much later, often during the

night. Furthermore, it was noted that oviposition often occurred within a few minutes of stress ending. Transport simulation was not used in subsequent experiments in this thesis as there were practical difficulties with recording oviposition time during this.

The aim of the following study was to describe the duration of oviposition delays caused by social stress and their consequences for eggshell quality with respect to specific abnormalities. A second aim was to confirm that relocating hens from individual cages to group cages would induce oviposition delays, as reported by Watt (1989), and that this manipulation could be used as a standard stressor in subsequent experiments in this thesis. In Experiment 1, hens were exposed to periods of stress which started and finished at various times relative to predicted oviposition time, thus allowing the distribution of oviposition times during stress to be described and some information to be obtained about the effects of stress ending after various durations of oviposition delay. Experiment 1 suggested that, for ovipositions not occurring during exposure to stress, the duration of oviposition delay (from expected oviposition time) at the time of stress ending influenced the time from stress ending until observed oviposition time. This relationship was investigated further in Experiment 2.

## 3.2 Experiment 1: Duration of Oviposition Delays Caused by Exposure to Social Stress and Consequences for Eggshell Quality

### 3.2.1 Materials and Methods

From a stock of 78 hens, a total of 67 whose next oviposition times could be predicted were selected over 7 days when they were between 43 and 52 weeks of age. Selected hens were housed together in groups of 4 in an unfamilliar cage (social stress) for 3-6 h, starting 0-4.5 h before predicted oviposition time (an unselected stock bird was used to complete the final group of 4).

Eggs laid on the day of exposure to stress and on 2-11 days (mean = 7) adjacent to this (control eggs) were classified as white-banded, slab-sided or dusted/normal, as defined by Hughes *et al.* (1986). The dusted/normal classification included eggs with no obvious dusting and those with a clearly visible layer of additional calcification. The level of eggshell dusting on dusted/normal eggs was measured by reflectometry at the centre of the broad pole, the point at which this coating is greatest (Mills *et al.*, 1991), as described by Mills *et al.* (1987a). Briefly,

the reflectance (of light) of the dry shell was measured using a battery powered eggshell reflectometer (Sharrard Developments Ltd., York). The eggshell was then moistened, using a damp swab, to make the layer of additional calcification transparent and so reveal the darker colour of the underlying eggshell. Wet shell reflectance was measured and deducted from the dry shell reflectance to give the dusting score. For heavily dusted eggs, the difference between wet and dry reflectance measurements is high. It was clear that the level of dusting in the absence of stress (control eggs) varied greatly between hens. To account for this, the change in dusting associated with exposure to stress was calculated for each hen by deducting the basal level of dusting (mean dusting score of control eggs) from the dusting score of the egg laid on the day of stress.

### 3.2.2 Results

*Duration of Oviposition Delays*

For 17 of the 67 hens, expected oviposition time could not be accurately calculated because records of oviposition times were incomplete (due to failures of the automatic oviposition recording equipment, see Section 2.2), or because exposure to stress was within three days of the egg sequence ending. Consequently, these hens were excluded from the following analysis. Of the remaining 50 hens, 34 laid during stress while 16 did not and still carried their eggs when they were returned to their original cages (i.e. when stress ended).

Thus, delaying of oviposition during stress ended either because oviposition occurred during stress or because exposure to stress ended. Both these events occurred at various times relative to expected oviposition time. In order to describe the distribution of oviposition times during exposure to stress, instances where exposure to stress ended before oviposition occurred (and where the actual time when these birds would have laid if stress had continued is not known) must be accounted for. This was done by calculating the "survivor function", an analysis which describes the probabilities of oviposition occurring during stress relative to time after expected oviposition time.

Calculation of survivor functions is described by Kalbfleisch and Prentice (1980). Briefly, as the duration of exposure to stress increases relative to expected oviposition time, the number of hens which have not yet laid and are still exposed to stress decreases when a hen either lays or is removed from stress. For each of these events the proportion of hens which could have laid but did not was calculated, i.e. the probability of an individual laying at this event (probability = number of hens

carrying an egg after the event / number of hens carrying an egg before the event). At the time of each event the probability of an individual hen laying at or before this event was calculated (probability = probability of laying at 1st event x probability of laying at 2nd event... x probability of laying at latest event). Thus, the survivor function describes the probability that an individual hen would still be carrying its egg after various durations of exposure to stress relative to expected oviposition time (Figure 3.1). From this function it can be seen that the probability of an individual hen delaying oviposition for more than 1 h (for example) during continued exposure to stress was 0.6. The maximum oviposition delay for a hen laying during stress was 3.0 h.

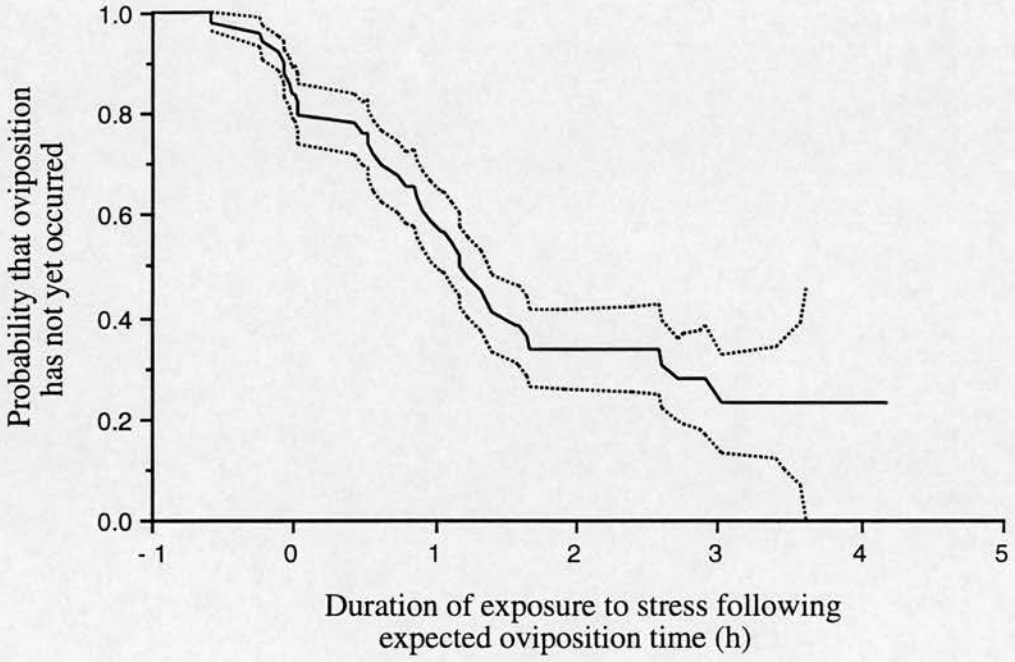



**Figure 3.1** Survivor function for Experiment 1, describing the relationship between the probability that an individual hen will not yet have laid during exposure to stress and the duration of exposure to stress after expected oviposition time. The survivor function +1 SEM and the survivor function -1 SEM are shown by the upper and lower dotted lines respectively. As duration of stress increases, sample size decreases (because hens either lay or their exposure to stress ends) and so SEM increases.

For the 16 hens not laying during exposure to stress, the relationship between the time when stress ended and the time when oviposition occurred is shown in Figure 3.2. Hens usually laid shortly (< 2 h) after exposure to stress ended when this occurred close to expected oviposition time, i.e. when oviposition delays were still relatively short. In contrast, when stress did not end until several hours after expected oviposition time, hens more often held their eggs until much later (> 2 h after stress ending). The shortest delay beyond which a hen did not lay < 2 h after stress ending was 2.4 h and the longest delay beyond which a hen laid < 2 h after stress ending was 3.6 h. Thus, oviposition delays ended either a short time after stress ending or not until much later and this may be related to a threshold of between 2.4 h and 3.6 h oviposition delay. The hypothesis that hens become less able to lay their eggs < 2 h after stress ending when oviposition delay exceeds 2.4 h was tested in Experiment 2.

For 7 of the 8 hens laying shortly after stress ended, oviposition occurred 8-22 min (mean = 16 min) after stress ending (Figure 3.2). Although the 8th hen was recorded as laying 105 min after stress ending, this may be an overestimate as clocks triggered by eggs rolling from cages were used to automatically record oviposition times and this egg might not have rolled from the cage until some time after it was laid (see Section 2.2). For these 8 hens, the maximum oviposition delay was 3.8 h.

For the 8 hens not laying < 2 h after stress ending, oviposition took place much later. One hen's egg did not roll from the cage and so observed oviposition time was not recorded. For this hen, stress ended 2.9 h after expected oviposition time and oviposition delay was > 5 h. Hen #48 laid after a delay of 23.6 h, at the expected oviposition time of the next day's egg. There was no other oviposition by hen #48 on the day after exposure to stress, although a single normal egg was laid on each of the subsequent days. The times of these subsequent ovipositions suggest that the egg on the day after stress was ovulated internally. For the other 6 hens, duration of oviposition delay was 7.1-15.2 h (mean = 12.3 h).

Oviposition delays were classified as short-term if they were less than 5 h in duration or long-term if they were longer than this. Therefore, oviposition delays ending during exposure to stress were considered to be short-term. Although behaviours other than oviposition were not recorded systematically, it was noted that hens often adopted a characteristic hunched, tail down posture when oviposition delays did not end shortly after stress ending (long-term delays).

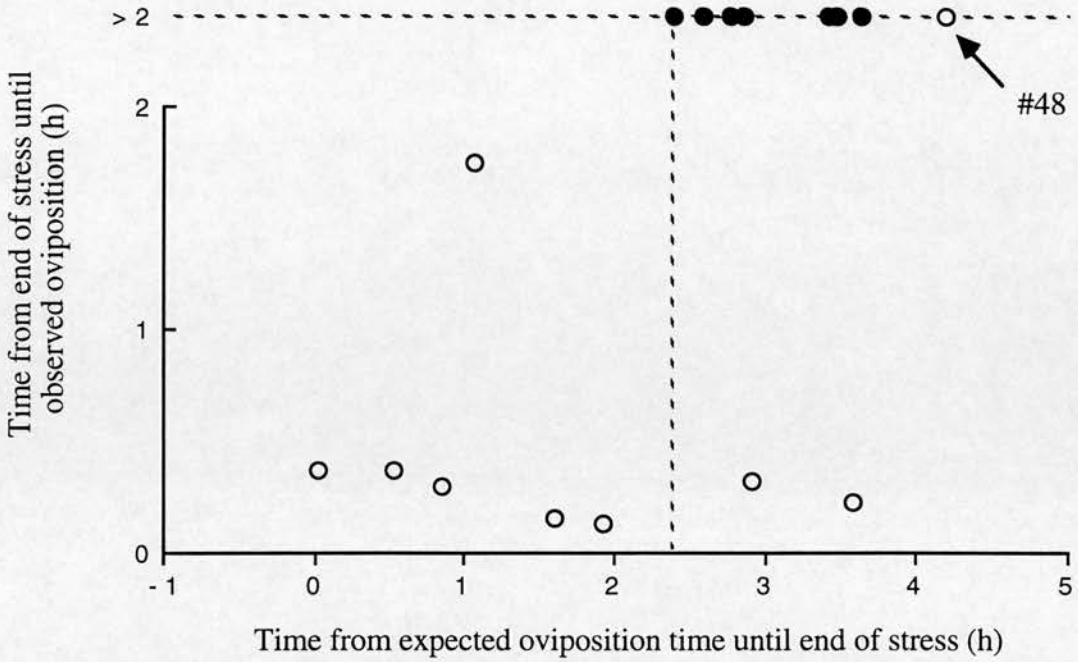



**Figure 3.2** Relationship between the time from expected oviposition time until end of stress (i.e. the duration of oviposition delay when stress ended) and the time from stress ending until observed oviposition time. Only hens not laying during stress in Experiment 1 are shown. The vertical dashed line indicates 2.4 h, the time beyond which hens appear less likely to lay < 2 h after stress ending (see text p. 34). Solid circles represent white-banded eggs and open circles represent eggs with normal or dusted shells. The egg laid by hen #48 is indicated.

*Eggshell Abnormalities*

Of the eggs laid following long-term oviposition delays, all but that of hen #48 were white-banded (Figure 3.2), and in each case the subsequent egg was slab-sided. Two of these slab-sided eggs were laid prematurely and were soft-shelled. The delayed egg of hen #48 was heavily dusted (score = 8.11) compared with non delayed eggs of this hen (mean score = 0.8). Short-term oviposition delays did not cause slab-side or white-band eggshell abnormalities.

One hen's eggs were always speckled, preventing accurate measurement of dusting, so were not included in the following analysis. Table 3.1 shows the levels of eggshell dusting associated with short-term stress-induced oviposition delays. Possible relationships between the duration of oviposition delay, the level of

eggshell dusting on the delayed egg (i.e. the egg laid on the day of stress) and the basal level of eggshell dusting were examined (not including eggs delayed long-term). The level of dusting on the delayed egg was significantly and positively correlated with the duration of oviposition delay (Spearman rank correlation coefficient ($r_S$)=0.342, n=41, $P<0.05$). There was also a correlation between the duration of oviposition delay and the change in dusting level, relative to the basal level, ($r_S$=0.381, n=41, $P<0.05$). Basal level of eggshell dusting was significantly and positively correlated with the level of eggshell dusting on the egg laid on the day of stress ($r_S$=0.649, n=41, $P<0.01$) but not with the change in dusting level of this egg, relative to the basal level, ($r_S$=0.020, n=41, $P>0.05$). When analysing possible relationships between the basal level of eggshell dusting and the duration of oviposition delay, hens that laid after stress ended were excluded as their duration of delay was determined by the stress ending. Duration of oviposition delay and basal level of dusting were not significantly correlated ($r_S$=0.048, n=34, $P>0.05$).

| Duration of oviposition delay for egg laid on day of stress | Number of hens | Mean basal level of dusting (± SEM) | Mean dusting of eggs laid on day of stress (± SEM) |
|---|---|---|---|
| < 0.5 h | 13 | 0.8 (0.1) a | 1.3 (0.2) b |
| 0.5-1.5 h | 19 | 1.2 (0.2) a | 2.0 (0.3) c |
| > 1.5 h | 9 | 1.3 (0.4) a | 3.1 (0.9) bc |

**Table 3.1** Effects of short-term oviposition delays, of either < 0.5 h, 0.5-1.5 h or > 1.5 h duration, on eggshell dusting in Experiment 1. Within rows (paired t-test) and within columns (t-test), values with no common letter differ significantly ($P<0.05$).

## 3.3 Experiment 2: Stress-Induced Oviposition Delays: Duration, Effects of Exposure to Stress Ending and Consequences for Eggshell Quality

Experiment 1 suggested a relationship between the duration of oviposition delay when stress ends and the time from stress ending until oviposition. When oviposition delay was < 2.4 h, oviposition usually occurred about 16 min after stress ended. In contrast, when oviposition delay was > 2.4 h, eggs were usually laid

several hours after stress ending. The aim of Experiment 2 was to test the hypothesis that oviposition will tend to occur < 2 h after stress ending if oviposition delay is currently < 2.4 h and will tend to occur > 2 h after stress ending if oviposition delay is already > 2.4 h. Hens were exposed to social stress for 0.33-6 h starting 1 h before predicted oviposition time. Thus, it was expected that hens would be returned to their home cages (when stress ended) with oviposition delays of various durations, including some < 2.4 h and some > 2.4 h. Unlike Experiment 1, the time from the start of stress until predicted oviposition time was constant in Experiment 2.

### 3.3.1 Materials and Methods

From 53 stock hens aged 26 weeks, 47 whose next oviposition times could be predicted were selected over four days and exposed to social stress starting 1.0 h before predicted oviposition time. Each hen was randomly allocated to receive one of 47 possible durations of stress. These were 20, 30, 40 min... until 210 min (i.e. 10 min intervals), then 215, 220, 225 min... until 330 min (i.e. 5 min intervals), then 340, 350 and 360 min. Experiment 1 suggested that a threshold oviposition delay at the time of stress ending, in the range of 2.4-3.6 h, might influence the time of ovipositions occurring after stress ends. In the present experiment, it was hoped that by including more periods of stress of medium duration, information about this threshold would be maximised. Note that the predicted oviposition times of hens exposed to short periods of stress (< 60 min) were after stress was due to end. As several of the four hens in each group cage during stress were selected hens (the rest being unselected stock birds), and these were all due to be stressed for different periods, the composition of each group changed frequently.

Eggs laid on the day of exposure to stress, on the 3 days before this and on the 3 days after this (i.e. 6 control eggs per hen) were classified as either white-banded, slab-sided or dusted/normal. Shell dusting of dusted/normal eggs was measured, as described in Section 3.2.1.

### 3.3.2 Results

*Duration of Oviposition Delays*

One hen was excluded from the analysis because records of its oviposition times following treatment were incomplete. Of the remaining 46 hens, 27 laid during exposure to stress while 19 did not and still carried their eggs when exposure to stress ended. For these 19 hens, the relationship between the time from expected

oviposition time until end of stress and the time from the end of stress until observed oviposition time is shown in Figure 3.3. Numbers of ovipositions occurring either < 2 h or > 2 h after stress ended when oviposition was delayed either < 2.4 h or > 2.4 h at the time of stress ending are shown in Table 3.2. This distribution differs significantly from random (Fishers exact test; $P<0.05$), supporting the hypothesis, from Experiment 1, that oviposition is more likely to occur < 2 h after stress ending if it is currently delayed < 2.4 h and is more likely to occur > 2 h after if it is already delayed > 2.4 h. The longest oviposition delay beyond which oviposition occurred < 2 h after stress ended was 4.3 h and the shortest oviposition delay beyond which oviposition occurred > 2 h after stress ended was 1.5 h

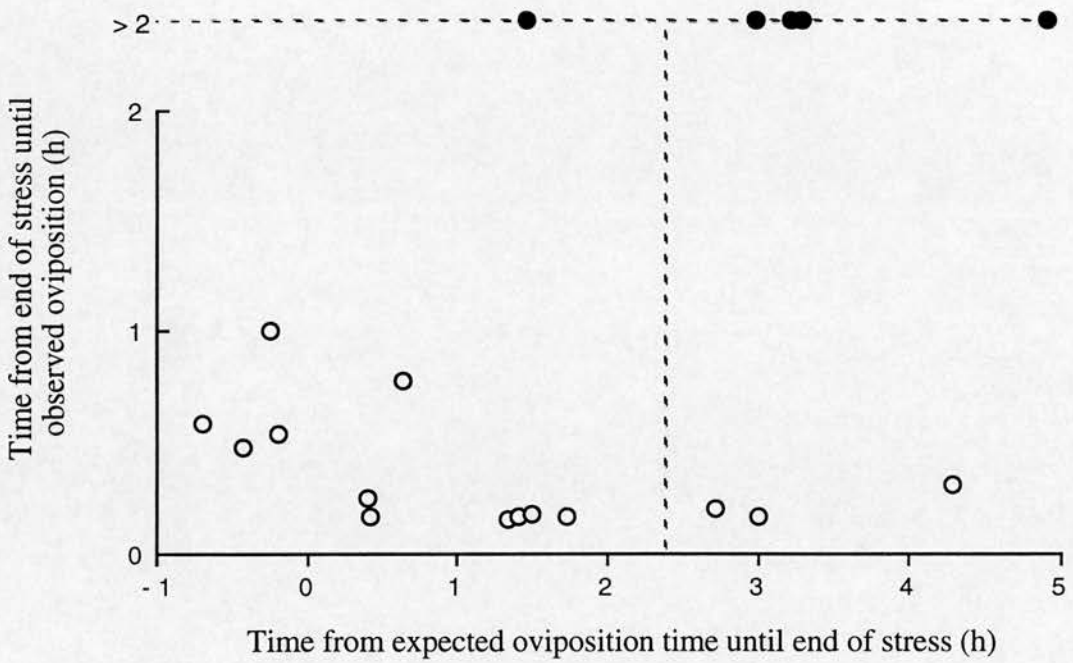



**Figure 3.3** Relationship between the time from expected oviposition time until end of stress (i.e. the duration of oviposition delay when stress ended) and the time from stress ending until observed oviposition time. Only hens not laying during stress in Experiment 2 are shown. The vertical dashed line indicates 2.4 h, the time beyond which hens appeared less likely to lay < 2 h after stress ending in Experiment 1. Solid circles represent white-banded eggs and open circles represent eggs with normal or dusted shells.

| Time from expected oviposition time until end of stress | Time from stress ending until observed oviposition | |
|---|---|---|
| | < 2 h | > 2 h |
| < 2.4 h | 11 | 1 |
| > 2.4 h | 3 | 4 |

**Table 3.2** Number of hens laying either < 2 h or > 2 h after stress ended when oviposition was delayed for either < 2.4 h or > 2.4 h at this time. Only hens laying after stress in Experiment 2 are included.

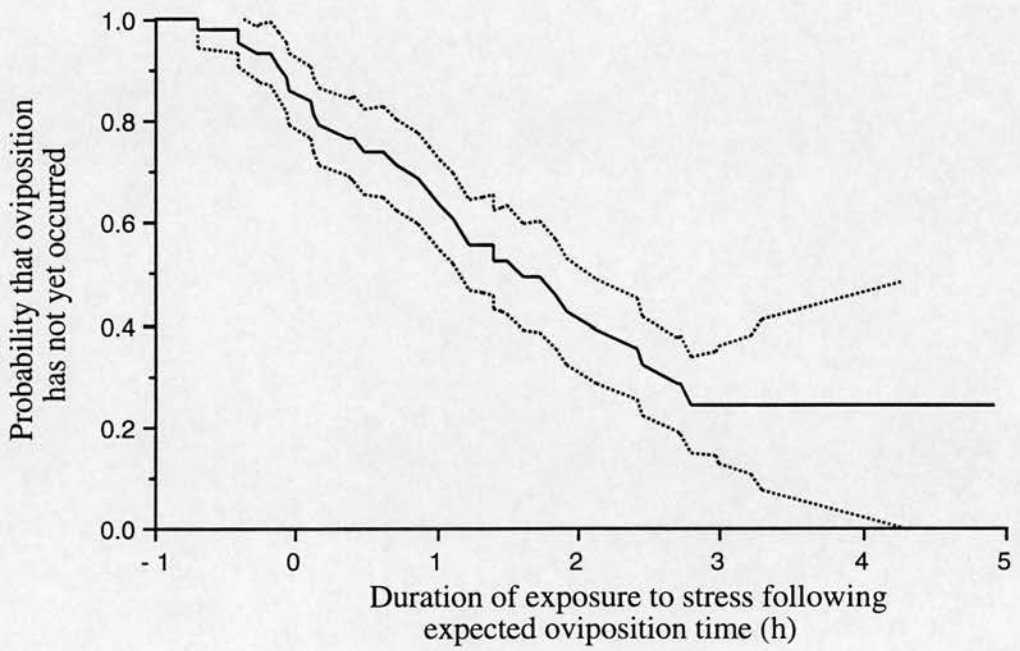



**Figure 3.4** Survivor function for Experiment 2, describing the relationship between the probability that an individual hen will not yet have laid during exposure to stress and the duration of exposure to stress after expected oviposition time. The survivor function +1 SEM and the survivor function -1 SEM are shown by the upper and lower dotted lines respectively. As duration of stress increases, sample size decreases (because hens either lay or their exposure to stress ends) and so SEM increases.

Observed oviposition times were only recorded for 2 of the 5 hens not laying within 2 h of stress ending. The durations of these oviposition delays were 11.0 h and 11.6 h. The other 3 eggs not laid within 2 h of stress ending were all delayed > 5 h.

The distribution of ovipositions occurring during stress is described by a survivorship function (Figure 3.4), as in Experiment 1. From this function, it can be seen that the probability of an individual hen delaying oviposition for more than 1 h (for example) during social stress was 0.6. The maximum oviposition delay for a hen laying during stress was 2.8 h.

Oviposition delays were classified as either short-term or long-term, as described for Experiment 1. As in Experiment 1, it was noted that hens tended to adopt a characteristic hunched, tail down posture when oviposition delays did not end shortly after stress ending.

*Eggshell Abnormalities*

All 5 eggs delayed long-term were white-banded when they were eventually laid (Figure 3.3) and in each case the next egg was slab-sided. Short-term oviposition delays did not cause either of these eggshell abnormalities.

Table 3.3 shows the levels of eggshell dusting associated with short-term stress-induced oviposition delays. Possible relationships between the duration of oviposition delay, the level of eggshell dusting on the delayed egg (i.e. the egg laid on the day of stress) and the basal level of eggshell dusting were examined (not including eggs delayed long-term). The duration of oviposition delay was not significantly correlated with either the level of eggshell dusting on the delayed egg ($r_S$=0.038, n=41, $P$>0.05) or the change in level of dusting on this egg, relative to basal levels, ($r_S$=0.006, n=41, $P$>0.05). Thus, although exposure to social stress increased eggshell dusting (Table 3.3), this was not correlated with the duration of oviposition delay. Basal level of eggshell dusting was significantly and positively correlated with the level of eggshell dusting on the egg laid on the day of stress ($r_S$=0.633, n=41, $P$<0.01) but not with the change in dusting level on this egg, relative to basal levels, ($r_S$=0.156, n=41, $P$>0.05). When analysing possible relationships between the basal level of eggshell dusting and the duration of oviposition delay, hens that laid after stress ended were excluded as their duration of delay was influenced by the stress ending. Duration of oviposition delay and basal level of dusting were not significantly correlated ($r_S$=-0.317, n=27, $P$>0.05).

| Duration of oviposition delay for egg laid on day of stress | Number of hens | Mean basal level of dusting (± SEM) | Mean dusting of eggs laid on day of stress (± SEM) |
|---|---|---|---|
| < 0.5 h | 13 | 0.8 (0.2) a | 1.2 (0.3) ab |
| 0.5-1.5 h | 13 | 0.9 (0.2) a | 1.9 (0.5) b |
| > 1.5 h | 15 | 0.9 (0.2) a | 1.9 (0.6) ab |

**Table 3.3** Effects of short-term oviposition delays, of either < 0.5 h, 0.5-1.5 h or > 1.5 h duration, on eggshell dusting in Experiment 2. Within rows (paired t-test) and within columns (t-test), values with no common letter differ significantly ($P<0.05$).

## 3.4 Discussion

In Experiments 1 and 2, relocating hens from individual cages to group cages containing unfamiliar hens caused many to delay oviposition. This is consistent with the findings of Hughes *et al.* (1986), Watt and Solomon (1988) and Watt (1989), who also found that relocation and contact with unfamiliar hens induced oviposition delays.

In both Experiments 1 and 2, exposure to social stress ended at different times relative to expected oviposition time, so hens that did not lay during stress were returned to their home cages with oviposition delays of various durations. In Experiment 1 these hens tended to lay < 2 h after stress ended if oviposition delay was still short (e.g. 1 h) but tended to lay > 2 h after stress ended if oviposition delay was long (e.g. 4 h). Consequently, it was hypothesised that there may be a threshold duration of oviposition delay beyond which eggs can no longer be readily laid. In Experiment 2, the proportion of hens laying < 2 h after stress ending was significantly affected by the duration of oviposition delay (either < 2.4 h or > 2.4 h), supporting hypothesis that oviposition becomes less likely after a threshold.

There was a range of oviposition delay durations within which eggs were either laid < 2 h or > 2 h of stress ending in both Experiment 1 (2.4-3.6 h) and Experiment 2 (1.5-4.3 h). Perhaps this reflects individual variation in the time of the

threshold. The hypothesis that hens become unable to lay readily following a threshold is supported by the observation that they did not lay during stress in either experiment after delays of more than 3.0 h. Although the threshold is clearly not described by a single time, for convenience it will be referred to as the 3 h threshold from here on.

Oviposition can be delayed surgically by fixing a thread around the utero-vaginal sphincter to obstruct the passage of the egg. When this thread is removed by a simple procedure 3 h after expected oviposition time, oviposition does not occur until much later (Tanaka, 1976; Tanaka *et al.*, 1984), supporting the hypothesis that a threshold restricts oviposition to within 3 h after expected oviposition time.

Oviposition delays can be classified as either short-term (those occurring before the threshold) or long-term (those occurring after the threshold). There appears to be a period of several hours between the threshold and the ovipositions of long-term delayed eggs when no eggs are laid, i.e. there were no medium duration oviposition delays.

In both experiments, ovipositions that were delayed usually occurred within 25 min after stress ending when this occurred before the threshold, indicating that the mechanisms underlying these delays can be reversed within this time. Kite (1985) also found that hens laid delayed eggs within a few minutes after stress ended (when closed nest boxes were opened). It is not surprising that oviposition did not occur soon after stress ending when this was earlier than expected oviposition time (Experiment 2), as this would have made them premature. Instead, these ovipositions occurred near their expected times. This suggests that only stress at the expected oviposition time will delay oviposition and is consistent with previous reports (Hughes *et al.*, 1986).

Ovipositions delayed beyond the 3 h threshold (long-term) eventually occurred after delays of 7.1-15.2 h (excluding hen #48) and were sometimes accompanied by premature oviposition of the next egg. A similar range of oviposition delays is caused by treatment with indomethacin, a PG synthesis inhibitor (Hertelendy & Biellier, 1978; Hiura *et al.*, 1982; Saito *et al.*, 1987; Balog & Hester, 1991). It is not known what causes oviposition of these eggs. Presumably, an increase in uterine contractions does not normally occur at this time, otherwise premature expulsion of the soft shelled eggs normally present would result. Irritation from a loop of thread sutured through the uterine wall causes premature oviposition (Sykes, 1953b; Lake & Gilbert, 1964; Ogasawara *et al.*, 1974). Perhaps the presence of two eggs in the uterus (the delayed egg and the subsequent egg) irritates the uterus, by over stretching it, and so leads to expulsion of the delayed

egg. These contractions might also account for instances where the subsequent egg was expelled prematurely.

In Experiment 1, hen #48 delayed oviposition for about 24 h and this egg was eventually laid at the expected oviposition time of the next day's egg. There was no evidence of this next egg, but the hen laid normally on subsequent days. Presumably the egg which would normally have joined the delayed egg in the uterus was ovulated internally and the uterine contractions for its oviposition expelled the delayed egg. This is consistent with the above hypothesis that contact between the delayed egg and the subsequent egg causes oviposition of the delayed egg.

An ovulation is not associated with the last oviposition of a sequence, with the first ovulation of the next sequence occurring one or more days later. Thus, if the oviposition of the last egg of a sequence was delayed then another egg would not join it in the uterus for at least a day. Uterine contraction frequency rises in association with the first ovulation of a sequence (Shimada & Asai, 1978), and this might expel a delayed egg. Indeed, when the last oviposition of a sequence is surgically delayed, by a temporary ligature closing the vagina, it eventually occurs in association with the first ovulation of the next sequence (Tanaka *et al.*, 1984). Hence, it is predicted that where the last oviposition of a sequence has been delayed, the delay will end in association with the first ovulation of the next sequence.

That a threshold prevents oviposition occurring after a delay of approximately 3 h has not previously been reported and therefore its function and underlying causation must be considered. In a normal egg cycle, increased uterine contractions during the period of eggshell formation might expel the egg prematurely and in a soft-shelled state. Such a loss of potential offspring would reduce genetic fitness, and so mechanisms may have evolved to prevent uterine contractions at inappropriate times. These might be responsible for the threshold beyond which delayed eggs are no longer expelled.

Although several mechanisms for the threshold can be hypothesised, evaluation of these is related to the understanding of the mechanisms underlying the stress-induced oviposition delays themselves. For this reason the following discussion is intended only to introduce these hypotheses and a more detailed evaluation of their merits is left until the General Discussion (Chapter 12).

Ovarian PG release, which causes uterine contractions and so initiates oviposition, may be controlled by endocrine changes of the ovulatory cycle (Etches *et al.*, 1990). Perhaps these endocrine changes define a window, with an upper limit

defined by the threshold, outwith which this PG release does not occur and hence oviposition is not initiated.

Although it is not known by how much the ovarian stores of PGs and their precursors are depleted in association with oviposition, about 30% of neurohypophysial AVT is released during a normal oviposition (Tanaka & Nakajo, 1962). Perhaps neurohypophysial AVT and/or ovarian PGs are released during stress-induced oviposition delays and the threshold reflects the time beyond which their stores are depleted and insufficient remains to cause oviposition.

In hens, oxytocin (and presumably AVT) inactivating enzyme activity of both uterine tissues and plasma is lowest at the time of normal oviposition and then rises within a few hours (Gilbert & Lake, 1964; Brzezinska *et al.*, 1967; Brzezinska-Slebodzinska *et al.*, 1979). Possibly, the threshold is when the half-life of AVT is reduced to the extent that any released is rapidly degraded and fails to induce oviposition.

Both AVT and PGF2$\alpha$ injections induce premature oviposition more potently when injected close to expected oviposition time, compared with other times in the egg cycle, and this may reflect changing uterine sensitivity to these hormones (Rzasa & Ewy, 1970; Hertelendy *et al.*, 1975; Goto *et al.*, 1985). Both the number of uterine AVT receptors and their binding characteristics vary during the egg cycle, under the influence of ovarian hormones, in such a way that the uterine contraction inducing potency of circulating AVT is greatest around the normal oviposition time (Takahashi *et al.*, 1992, 1994). The uterine PG receptors may also show similar changes (Asboth *et al.*, 1985). Perhaps the threshold is the time beyond which uterine sensitivity to AVT and/or PGs has reduced to the extent that these hormones can no longer cause oviposition.

Chapter 7 describes experiments which test the abilities of AVT and PGF2$\alpha$ injections to induce ovipositions delayed beyond the threshold. If AVT or PGF2$\alpha$ are unable to expel these eggs then this would support the hypotheses that the threshold was due to either reduced uterine sensitivity to AVT or PG or reduced AVT half-life.

In both experiments, many hens laid during exposure to social stress and there was much variation in the duration of their oviposition delays. Perhaps these hens became tolerant to the disturbance and so were able to lay their eggs. Some hens may be less susceptible to this form of stress, e.g. those which were dominant within their social group, and so delayed their oviposition for shorter periods.

All eggs delayed long-term, except that of hen #48, were white-banded and the egg following each was slab-sided, and also soft-shelled if its oviposition was premature. These abnormalities are caused by contact in the uterus between the delayed egg and the recently ovulated egg (van Middelkoop, 1971). As discussed already, the egg after the delayed egg of hen #48 may have been ovulated internally. Therefore the lack of white-banding on this egg's shell is because no egg pressed against it in the uterus. Some slab-sided eggs were laid prematurely, and consequently had soft shells, and this has been reported previously (van Middelkoop, 1971). Eggshell dusting, but not white-banding, often resulted from short-term oviposition delays. That long oviposition delays cause white-band and slab-side abnormalities while short delays only cause dusting is in agreement with the findings of Hughes *et al*. (1986).

It has been suggested that the maximum proportion of eggs that can be slab-sided is 50%, as each is paired with a white-banded egg (Ivy *et al.*, 1972). However, during preliminary investigations for the current experiments, eggs were collected which were both white-banded and slab-sided. Van Middelkoop (1971) also reported that eggs may have both slab sides and white bands. This indicates that oviposition of slab-sided eggs can also be delayed, and therefore more than 50 % of eggs could, in theory, be slab-sided.

Hens normally laying in nest sites typically do not lay white-banded eggs in these (van Middelkoop, 1971; R.Freir, personal communication; J.C.Cooper, personal communication), suggesting that these ovipositions are not accompanied by the pre-laying behaviours characteristic of normal oviposition. That delayed ovipositions occur in the absence of pre-laying behaviour has also been observed by Appleby (1986). Thus, in housing systems with nest sites, stress might cause some eggs not to be laid in these sites by delaying oviposition so that it occurs in the absence of pre-laying behaviour. Hence, oviposition delays could be one of the causes of floor eggs.

The hunched posture of hens delaying oviposition beyond the threshold may indicate abdominal pain (M.J.Gentle personal communication), possibly caused by the presence of two eggs in the uterus.

As eggshell dusting is associated with oviposition delays (Hughes *et al.*, 1986; Watt, 1989), and this deposition of additional calcium carbonate is presumably not instantaneous, a relationship between the duration of oviposition delay and the level of eggshell dusting may be expected. Although exposure to social stress increased the level of eggshell dusting in both Experiments 1 and 2, the level of

eggshell dusting, and the increase over basal levels that this represented, was correlated with the duration of oviposition delay only in Experiment 1. That hens in Experiment 1 (43-52 weeks old) were older than those in Experiment 2 (26 weeks old) may account for their different eggshell dusting responses to oviposition delay. Although level of eggshell dusting in the absence of stress may be lower in older hens (Mills *et al.*, 1991), there are no reports comparing the effects of age on the dusting caused by exposure to stress.

Hens that regularly lay dusted eggs are more fearful than hens which lay normal eggs (Jones & Hughes, 1986; Mills *et al.*, 1991). If fearful hens are more likely to delay oviposition when stressed, the duration of oviposition delay may be associated with the basal level of dusting. The present study does not support this suggestion as the duration of oviposition delay was not correlated with the basal level of eggshell dusting in either experiment.

In both Experiments 1 and 2, level of eggshell dusting on eggs laid on the day of stress was associated with the basal level of eggshell dusting. This is not surprising as hens normally laying heavily dusted eggs will presumably lay eggs with at least that level of dusting on the day of stress. Basal level of eggshell dusting was not correlated with the increase in eggshell dusting associated with stress, relative to basal levels, in either experiment, suggesting that hens with high basal levels of eggshell dusting do not lay down more additional calcium carbonate (dusting) when stressed than hens with low basal dusting levels.

As a short-term oviposition delay can cause an egg to become dusted while a long-term delay causes a white-banded egg and a slab-sided egg, the duration of delay affects both the number of abnormal eggs and the severity of these abnormalities. Consequently, the findings of this chapter may be of importance to the poultry industry. Firstly, it appears that only periods of stress which span expected oviposition times will cause oviposition delays, and this is consistant with the findings of Hughes *et al.* (1986). Therefore, restricting disturbances to the afternoon, when most hens will already have laid (Bahr & Johnson, 1991), should avoid causing delays and related eggshell abnormalities. Secondly, the 3 h threshold means that any unavoidable disturbances in the morning should be restricted to short periods, perhaps 1-2 h, separated by 1 h of no disturbance. As hens usually lay within 25 min of stress ending when oviposition delay is < 2 h, any ovipositions delayed by the disturbance should then occur during the periods of no disturbance.

From an animal welfare perspective, the present findings support the view (Hughes *et al.*, 1986; Mills *et al.*, 1987b, 1991) that certain eggshell abnormalities

are caused by environmental stress, and so may provide a means of detecting such disturbances. Although white-banded and slab-sided eggs clearly reflect oviposition delays, the association between dusting and stress is less robust as this defect can occur in the absence of stress and may be affected by the hen's age (Nys, 1991; Mills *et al.*, 1991).

# CHAPTER 4: Plasma PGFM and AVT Concentrations Associated with Stress-Induced Oviposition Delays

## 4.1 Introduction

Oviposition is initiated by the release into the circulation of PGs, including PGF2α, from the ovarian follicles (see Shimada & Saito (1989) for a review). These PGs increase the contraction frequency of uterine muscles and so trigger release of neurohypophysial AVT into the circulation. AVT further promotes uterine contractions, both directly and by stimulating synthesis of PGs within the uterine tissues themselves. The peripheral plasma concentrations of 13,14-dihydro-15-keto prostaglandin F2α (PGFM), a more stable but less active metabolite of PGF2α, and PGF2α are directly correlated (Olson & Hertelendy, 1981). Peripheral plasma PGFM concentration rises in association with oviposition and this is thought to reflect ovarian PGF2α release (Hammond *et al.*, 1980; Olson & Hertelendy, 1981; Olson *et al.*, 1986; Etches *et al.*, 1990). Plasma AVT concentration rises about four-fold in association with oviposition (Arad & Skadhauge, 1984; Tanaka *et al.*, 1984; Rice *et al.*, 1985; Shimada *et al.*, 1986; Koike *et al.*, 1988). This rise starts about 5 min before oviposition, reaches a maximum at the time of oviposition and then rapidly decreases in the following 5 min.

A variety of environmental stressors can cause hens to delay oviposition (Scott, 1940; Hughes, 1979; Hughes *et al.*, 1986; Watt, 1989). Several mechanisms by which stress might cause oviposition delays are hypothesised in Section 1.5. One hypothesis is that stress causes an opioidergic inhibition of neurohypophysial AVT release, a mechanism similar to that known to underlie stress-induced disruptions of parturition and lactation in mammals (e.g. Haldar & Bade, 1981; Petherick *et al.*, 1993). A second hypothesis is that stress may inhibit ovarian PG release.

The hypothesis that stress-induced oviposition delays are due to inhibition of AVT release leads to some predictions about the changes in plasma AVT concentration associated with these delays. Firstly, AVT concentration should not rise to the levels associated with normal oviposition during oviposition delays. If AVT concentration was to rise during delays then stress would not be inhibiting its release and another mechanism must be responsible for delaying oviposition. Secondly, AVT concentration should rise in association with delayed oviposition. If this does not occur then AVT would clearly not be essential for expulsion of the egg and therefore inhibition of AVT release would be an unlikely cause of delays. This possibility must

be considered as hens which have been neurohypophysectomised or treated with AVT antiserum lay normally and this has cast doubt on the importance of AVT in the oviposition process (Shirley & Nalbandov, 1956; Opel, 1965; Nakada *et al.*, 1993, 1994).

As ovarian PG release initiates oviposition and triggers AVT release (via uterine contractions), the hypothesis that stress-induced oviposition delays are due to inhibition of AVT release also allows predictions concerning plasma PG concentration. Firstly, PG concentration should rise at delayed oviposition. Secondly, PG concentration should rise during oviposition delays. If this stimulus for AVT release does not occur, the oviposition delays may not be due to inhibition of AVT release.

If the second hypothesis, that stress-induced oviposition delays are due to inhibition of ovarian PG release, is correct then PG concentration should rise at delayed oviposition but not during the delay. Furthermore, AVT concentrations should not rise during these delays as the stimulus for this, PG induced increase in uterine contraction frequency, would not occur.

The aim of this study was to measure the changes in plasma PGFM and AVT concentrations associated with stress-induced oviposition delays. In particular are these concentrations elevated at delayed oviposition or during the delays?

To fully test for possible rises in plasma PGFM or AVT concentration during oviposition delays, ensuring that any short duration rises are detected, would require frequent removal of blood samples. There are two reasons why this was not practicable. First, due to assay constraints, plasma PGFM and AVT concentrations could only be measured in a limited number of samples. Second, formation of haematoma often hinders serial sampling from wing veins. Although cannulating a wing vein would allow more samples to be taken, it is not known in advance which hens will be used in an experiment and therefore require this surgery. In the following experiments a single blood sample was taken during each oviposition delay. Thus, prolonged increases in hormone concentration should be detected, although short duration increases might not be.

Experiment 1 describes changes in plasma PGFM concentration associated with normal ovipositions and those delayed in response to social stress. In this experiment measurements of PGFM concentration associated with normal ovipositions were not directly comparable to those associated with delayed ovipositions and the PGFM assay was not fully validated. Therefore, although the data obtained are reported, further investigation involving a more balanced experimental design and a full validation of the assay will be required before firm conclusions can be made.

Experiment 2 describes changes in plasma AVT concentration associated with normal ovipositions and those delayed in response to social stress.

## 4.2 Experiment 1: Plasma PGFM Concentrations Associated with Stress-Induced Oviposition Delays

The aim of this experiment was to investigate plasma PGFM concentrations associated with stress-induced oviposition delays. In particular, is PGFM concentration elevated during these delays or at delayed oviposition?

### 4.2.1 Materials and Methods

From 35 stock hens aged 34 weeks, 9 whose next oviposition times could be predicted were selected. In order to measure plasma PGFM concentrations associated with normal oviposition, these hens were blood sampled 30 min before predicted oviposition time, immediately after observed oviposition and 30 min after observed oviposition (control treatment). Over three subsequent days, a further 13 hens whose next oviposition times could be predicted were selected. In order to induce oviposition delays, these hens were each exposed to a period of social stress starting 45 min before predicted oviposition time (stress treatment). Blood samples were taken 30 min before predicted oviposition time (during stress) and 30 min after predicted oviposition time (during oviposition delay and during stress). Social stress was ended after the second blood sample by returning each hen to its original cage. From Chapter 3, it was expected that these hens would lay about 15 min after stress ended. A final blood sample was taken immediately after these delayed ovipositions.

Blood samples were placed in micro-centrifuge tubes containing aspirin and citrate (a prostaglandin synthesis inhibitor and an anti-coagulant, respectively; see Section 2.6) and these were separately prepared for control and stress treatments. Plasma PGFM concentrations were determined in duplicate by R.W.Kelly using the assay procedure described by Harrison (1990). This assay has been validated for use in human but not avian plasma. Therefore, to confirm a linear relationship between actual and measured PGFM in avian plasma, stock plasma (taken immediately after oviposition and therefore expected to have a high PGFM concentration) was serially diluted with 0.9% saline.

Mean PGFM concentrations were compared between treatments by t-test (pooled where standard deviations were similar) and between blood sampling times within each treatment by paired t-test.

### 4.3.2 Results

Of the 13 hens exposed to social stress, 4 laid during stress and before 30 min after predicted oviposition time and so were excluded from the assay and the analysis (thus, n=9 for stress and control treatments).

The changes in mean plasma PGFM concentration associated with ovipositions of undisturbed hens and those of hens exposed to social stress are shown in Table 4.1. Although PGFM concentration was higher immediately after oviposition than at other sampled times for control hens, this was not significant. For the stress treatment, PGFM concentration was significantly higher at oviposition than at other

| Treatment | Blood sample | Minutes after expected oviposition time. Mean (±SEM) | ng/ml PGFM. Mean (±SEM) |
|---|---|---|---|
| control | before oviposition | -32.8 (5.9) | 16.8 (13.4) abc |
| control | immediately after oviposition | 1.4 (5.4) | 32.4 (10.1) a |
| control | after oviposition | 27.2 (5.5) | 14.4 (3.2) ab |
| stress | before expected oviposition time | -28.4 (5.2) | 2.8 (0.7) c |
| stress | during oviposition delay | 33.3 (5.4) | 7.7 (1.4) b |
| stress | immediately after oviposition | 49.6 (4.3) | 74.4 (13.9) d |

**Table 4.1** Mean blood sampling times and mean plasma PGFM concentrations associated with ovipositions of undisturbed hens (control, n = 9) and ovipositions of hens exposed to social stress (stress, n = 9). Mean PGFM concentrations with a common letter do not differ significantly ($P>0.05$).

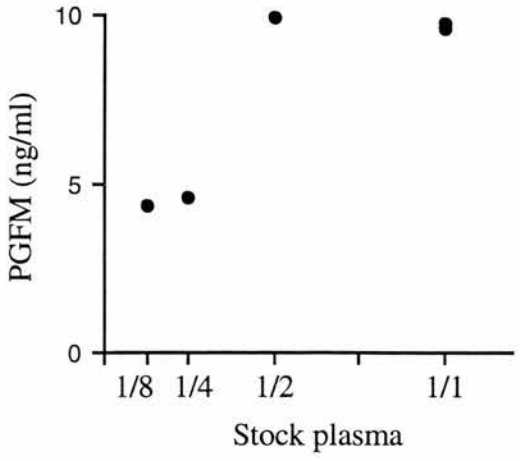



**Figure 4.1** Measured PGFM concentrations for plasma serially diluted with saline. concentration was measured twice for undiluted plasma.

times. PGFM concentration was significantly higher at delayed oviposition (stress treatment, mean oviposition delay = 48 min) than at normal oviposition (control treatment, mean oviposition delay = 0 min). During oviposition delays PGFM concentration was significantly higher than before expected oviposition time.

For the control treatment, the plasma PGFM concentration in one blood sample taken 30 min before predicted oviposition time (123.8 ng/ml) was much higher than in the other 8 samples taken at this time (mean = 3.4 ng/ml, SEM = 0.9). If this high value is omitted, mean PGFM concentration before oviposition in control birds is significantly lower than that at oviposition or at 30 min after predicted oviposition time for both stress and control treatments. This mean PGFM concentration (3.4 ng/ml) is similar to that for blood samples taken at the equivalent time (i.e. 30 min before predicted oviposition time) from hens exposed to stress (2.8 ng/ml).

Halving dilutions of plasma with saline did not have a linear effect on PGFM concentration (Figure 4.1).

## 4.3 Experiment 2: Plasma AVT Concentrations Associated with Stress-Induced Oviposition Delays

The aim of this experiment was to investigate plasma AVT concentrations associated with stress-induced oviposition delays. In particular, is AVT concentration

elevated during these delays or at delayed oviposition? The design of this experiment was improved, relative to Experiment 1, by randomly allocating hens between control and stress treatments each day, so that data were balanced for potential day effects, and by changing the time when stress ended (see below). The assay used to determine AVT concentrations is validated in Appendix 2.

### 4.3.1 Materials and Methods

From 48 stock hens aged 25 weeks, 5-7 whose next oviposition times could be predicted were selected on each of four days and randomly (but unevenly) divided between control (n = 2-3 each day; total = 10) and stress (n = 3-5 each day, total = 15) treatments. Except for the removal of three blood samples, one 30 min before predicted oviposition time, one immediately after oviposition and one 30 min after oviposition, hens of the control treatment were not disturbed to allow oviposition to occur normally.

Hens allocated to the stress treatment were each exposed to a period of social stress starting 45 min before and ending 45 min after predicted oviposition time (thus, total duration = 90 min) in order to induce oviposition delays. From Chapter 3, it was expected that many of these hens would lay about 15 min after stress ended, i.e. with oviposition delays of around 60 min duration. Since some hens were expected to lay during exposure to stress, more were allocated to the stress treatment than to the control treatment each day. Blood samples were taken from these hens 30 min before predicted oviposition time (during stress), 30 min after predicted oviposition time (during oviposition delay and during stress) and immediately after ovipositions occurring when stress ended. If stress had ended immediately following the second blood sample and oviposition occurred soon after, it would not be known if this oviposition would have occurred had the stress continued. Therefore, it would not be possible to dissociate any high AVT concentration in a second blood sample from the oviposition. It was in order to avoid this problem that stress was continued for 15 min beyond the second blood sample (i.e. until 45 min after predicted oviposition time).

For 1 of the 10 hens allocated to the control treatment, the blood sample immediately after oviposition was not taken. For a second hen, blood sample volumes were small. For a third hen, expected oviposition time (retrospectively estimated) was later than predicted oviposition time (prospectively estimated) so that the first blood sample was taken only 2 min before expected oviposition time. As plasma AVT concentration could only be measured for a limited number of blood samples, those from these 3 hens were excluded. Of the 15 hens allocated to receive social stress, 4

laid during the stress period (mean oviposition delay = 36 min), 2 did not carry hard-shelled eggs on the day of treatment (possibly due to internal ovulation) and, for a seventh hen, haematoma formed during removal of the first blood sample and prevented removal of subsequent samples. For 2 of the 8 remaining hens, expected oviposition time was more than 30 min before predicted oviposition time so that the second blood samples were earlier than, and the stress periods ended close to (11 min before and 8 min after), expected oviposition time. These 9 hens were excluded from the assay and the analysis.

Thus, plasma AVT concentration was measured in blood samples from 7 control treatment and 6 stress treatment hens. These hens represented each treatment on each day. Plasma AVT concentrations were measured using an AVP radioimmunoassay kit as described in Appendix 2. Results presented are corrected for extraction efficiency.

Mean AVT concentrations were compared between treatments by t-test (pooled where standard deviations were similar) and between blood sampling times within each treatment by paired t-test.

### 4.3.2 Results

Changes in mean plasma AVT concentration associated with ovipositions of control hens and those of hens exposed to social stress are shown in Table 4.2. For one hen (stress) the first blood sample was not included in the analysis as it was taken 8 min after expected oviposition time. For control hens, AVT concentration was five-fold higher at oviposition compared to 30 min earlier and returned to basal levels within 30 min. Mean AVT concentration at delayed oviposition (stress treatment, mean oviposition delay = 54 min) was similar to that at normal oviposition (control treatment, mean oviposition delay = 1 min). Mean plasma AVT concentration during oviposition delay was not elevated relative to other non-oviposition times. There is no evidence to suggest that AVT concentration was elevated in any of the blood samples taken during oviposition delays (maximum AVT concentration = 41.6 pg/ml). Social stress did not alter mean AVT concentration at 30 min before expected oviposition time.

| Treatment | Blood sample | n | Minutes after expected oviposition time. Mean (±SEM) | pg/ml AVT. Mean (±SEM) |
|---|---|---|---|---|
| control | before oviposition | 7 | -30.6 (3.9) | 31.8 (4.4) a |
| control | immediately after oviposition | 7 | 2.3 (5.6) | 158.4 (15.6) b |
| control | after oviposition | 7 | 31.1 (5.1) | 30.6 (3.8) a |
| stress | before expected oviposition time | 5 | -30.6 (5.3) | 30.5 (6.1) a |
| stress | during oviposition delay | 6 | 33.7 (7.6) | 27.4 (5.3) a |
| stress | immediately after oviposition | 6 | 54.7 (4.5) | 157.5 (14.1) b |

**Table 4.2** Mean blood sampling times and mean plasma AVT concentrations associated with ovipositions of undisturbed hens (control) and ovipositions of hens exposed to social stress (stress). Mean AVT concentrations with a common letter do not differ significantly ($P>0.05$). Mean AVT concentrations with no common letter differ significantly ($P<0.01$).

## 4.4 Discussion

In Experiment 1, control and stress treatments were carried out on separate days and for each micro-centrifuge tubes containing citrate and aspirin were separately prepared. Therefore, potential effects of day and micro-centrifuge batch were not controlled for in the comparison of stress and control treatments. Furthermore, halving dilutions of plasma did not produce a linear change in PGFM concentration and so the use of this assay for measuring PGFM in avian plasma was not validated. Given these problems, the results must be interpreted cautiously and a further experiment with a validation of the assay will be required before firm conclusions can be made.

In control hens, PGFM concentration was elevated, although not significantly, at the time of oviposition. That PGFM concentration rises in association with

oviposition has been reported previously (Hammond *et al.*, 1980; Olson & Hertelendy, 1981; Olson *et al.*, 1986; Etches *et al.*, 1990). PGFM concentrations recorded here, both before (16.8 ng/ml) and at oviposition (32.4 ng/ml), were much higher than those reported previously, e.g. 0.27 ng/ml before oviposition and 1.75 ng/ml at oviposition (Olson & Hertelendy, 1981). In the present study PGFM concentration was lower at 30 min before normal oviposition than at 30 min after normal oviposition (control treatment), and this is consistent with previous reports (Olson & Hertelendy, 1981).

For hens exposed to stress, PGFM concentration was higher at delayed oviposition than during the delays or before expected oviposition time, presumably reflecting ovarian PG release in association with these ovipositions. This is consistent with the view that PG release causes oviposition (Shimada & Saito, 1989). During oviposition delays, PGFM concentration was higher than before expected oviposition time, and this may be due to ovarian PG release. The hypothesis that stress delays oviposition by inhibiting AVT release predicts that ovarian PG release, indirectly the stimulus for AVT release, should occur during these delays. The finding that PGFM concentration was elevated during oviposition delays, relative to before expected oviposition time, is consistent with this hypothesis. However, compared with the control treatment, PGFM concentration at 30 min after expected oviposition time was lower in hens exposed to stress. Due to the experimental design, the contribution of factors other than treatment, e.g. micro-centrifuge batch, towards this difference cannot be assessed. If stress does reduce plasma PGFM concentration then oviposition delays may be due to inhibition of ovarian PG release. Although PGFM concentration was significantly higher at delayed oviposition than at normal oviposition, it is not clear why this should be so, although factors other than treatment may again contribute towards this difference.

In control hens, AVT concentration rose five-fold in association with normal oviposition and this is consistent with previous reports (Arad & Skadhauge, 1984; Tanaka *et al.*, 1984; Rice *et al.*, 1985; Shimada *et al.*, 1986; Koike *et al.*, 1988). AVT concentrations recorded here, both before (31.8 pg/ml) and at oviposition (158.4 pg/ml), were higher than those reported previously, e.g. about 20 pg/ml before oviposition and about 80 pg/ml at oviposition (from the figures of Tanaka *et al.* (1984)). For hens exposed to stress, AVT concentration rose in association with delayed oviposition, reaching concentrations similar to those associated with normal oviposition. Therefore, AVT release may be an important component of the oviposition process. AVT concentration was not elevated in any of the blood samples taken during oviposition delays, supporting the hypothesis that stress causes

oviposition delays by inhibiting AVT release. In mammals, stress-related disruptions to parturition and lactation are due to opioidergic inhibition of oxytocin release (e.g. Leng & Russell, 1989). Stress may similarly inhibit AVT release in hens and so delay oviposition. The alternative hypothesis, that delays are due to inhibition of ovarian PG release, also predicts that AVT release will occur at delayed oviposition but not during the delay (see Introduction). Indeed, any oviposition delaying mechanism which prevents the stimulus for AVT release (PG induced uterine contractions), e.g. direct adrenergic suppression of uterine contractions, should cause the observed changes in AVT concentration. Therefore, the present findings are also consistent with the hypothesis that stress causes oviposition delays by preventing the stimulus for AVT release, i.e. PG induced uterine contractions.

In this study a specific type of stress-induced oviposition delay was considered, i.e. around 55 min duration with oviposition occurring about 15 min after stress ending, and this warrants justification. This was the shortest duration of delay during which a blood sample could be taken about 30 min after expected oviposition time. If stress was continued for longer than 30 min (Experiment 1) or 45 min (Experiment 2) past predicted oviposition time, then a greater proportion of hens would be expected to lay during stress. Blood samples were taken at delayed ovipositions which occurred shortly after stress ending rather than those occurring during stress for practical reasons. It was not possible to continually observe hens during stress in order to remove blood samples immediately after oviposition. Ending stress, allowing oviposition to occur about 15 min later, made oviposition time more predictable, and it was this predictability that allowed delayed ovipositions to be sampled accurately.

To summarise, plasma PGFM concentration was elevated in association with both normal ovipositions and those delayed for about 50 min in response to social stress. For hens exposed to stress, plasma PGFM was significantly higher during oviposition delays than before expected oviposition time, possibly reflecting release of ovarian PGs and so supporting the hypothesis that stress-induced oviposition delays are due to inhibition of AVT release. However, further investigation and validation of the assay will be required to verify these findings. Plasma AVT concentration was elevated in association with both normal ovipositions and those delayed for about 50 min in response to social stress and there was no evidence of elevated plasma AVT concentration during oviposition delays. This suggests that oviposition delays are due to inhibition of AVT release and/or the stimulus for this, i.e. PG induced uterine contractions.

# CHAPTER 5: **Recording Uterine Contractions *in vivo* with Extraluminal Strain Gauge Transducers**

## 5.1 Introduction

Uterine contraction frequency varies in a characteristic manner during the daily egg laying cycle (Shimada & Asai, 1978; Shimada & Etches, 1986; also see Section 1.4.1). In the 5-6 h after oviposition, while there is no egg in the uterus, contraction frequency is low (< 5 per min). Once an egg enters the uterus, contraction frequency increases to about 10 per min and this is maintained until just before oviposition. At that time contraction frequency rises two-fold, to about 20 per min, and this causes expulsion of the egg in about 3 min. Ovarian PGs and neurohypophysial AVT are released in association with oviposition and their elevated plasma concentrations are thought to cause the increase in contraction frequency (Shimada & Saito, 1989). Indeed, a two-fold rise in contraction frequency, similar to that associated with normal oviposition, accompanies ovipositions induced prematurely by injection of PGF2$\alpha$ (Shimada & Asai, 1979).

Several lines of evidence suggest that ovarian PG release causes an increase in uterine contraction frequency which then triggers AVT release (see review by Shimada and Saito (1989) and Chapter 1 p.14). This AVT acts to further promote uterine contractions, both directly and by stimulating PG production within the uterine tissues. As contractions (induced by PGs) act as the stimulus for AVT release and are also caused by the AVT once it is released, it is not known how much, or for how long, contraction frequency must be elevated to stimulate AVT release. Although investigating uterine contraction frequencies of neurohypophysectomised hens (in which AVT release is not thought to occur; see Chapter 1 p.14 & 15) might be a useful way to describe this trigger for AVT release, these experiments have not yet been reported. As AVT presumably causes further AVT release by inducing uterine contractions, a positive feedback loop is formed and is thought to have a cascading effect on the development of uterine contractions (see review by Shimada and Saito (1989) and Chapter 1 p.10).

A variety of stressors, such as relocation to novel environments and exposure to unfamiliar hens, can cause hens to delay oviposition (Scott, 1940; Hughes, 1979; Hughes *et al.*, 1986; Watt, 1989). One hypothesis to explain these delays is that AVT is important for expelling the egg and a stress-related opioidergic inhibition of AVT release prevents oviposition (see Chapter 1). Thus, it is suggested that stress disrupts the AVT-related escalation of uterine contraction frequency that follows, and is

initiated by, a rise in uterine contraction frequency induced by ovarian PGs. This hypothesis predicts that an increase in uterine contraction frequency, the stimulus for AVT release, should occur at the expected oviposition time when oviposition is delayed in response to stress. If this stimulus does not occur, then opioidergic inhibition of AVT release may not be the primary cause of stress-related oviposition delays. Instead, this would suggest an alternative hypothesis in which stress acts to prevent the stimulus for AVT release. Therefore if an increase in uterine contraction frequency during oviposition delay, which may be the stimulus for AVT release, was identified then this would support the hypothesis that inhibition of AVT release causes the delays.

The aim of this study was to investigate the uterine contraction frequencies associated with stress-induced oviposition delays. In particular, is there any evidence to suggest that frequency is elevated at expected oviposition time, i.e. is the stimulus for AVT release present?

Muscle contractions can be monitored by recording electromyographic (EMG) activity with implanted electrodes and this technique has been used to investigate the frequency of uterine muscle contractions in hens (e.g. Shimada, 1978; Roche & Brard, 1978). Contractions can also be monitored by directly recording movements of the muscle. Strain gauge transducers (SGTs) have been used to directly record contractions of uterine muscle in rabbits (Hawk *et al.*, 1982), ewes (Hawk & Conley, 1985) and cows (Burton *et al.* 1987). Although SGTs have been used to record contractions of various sections of the avian gut (Duke & Kostuch, 1975, Savory *et al.*, 1981), their use for recording avian uterine contractions has not been reported.

As the expertise and equipment for recording muscle contractions by SGT was available, this technique was used to monitor the frequency of uterine contractions. As this use of SGTs has not previously been reported, a second aim of this study was to assess the suitability of SGTs for recording avian uterine contractions. Unfortunately, the SGTs used had fragile wires which often broke preventing the recording of uterine contractions in many hens. For this reason a full investigation of the changes in uterine contraction frequency associated with oviposition delays was not possible and SGT recordings from only two hens are presented.

## 5.2 Changes in Uterine Contraction Frequency Associated with Normal Oviposition, Oviposition Delayed by Stress and Oviposition Induced Prematurely by PGF2α Injection

To validate the use of SGTs for recording uterine contractions, recordings associated with normal oviposition and oviposition induced prematurely by PGF2α injection were compared with published reports. Uterine contractions associated with oviposition delayed in response to environmental stress were then recorded.

### 5.2.1 Materials and Methods

Hens known to lay eggs in long and predictable sequences were used in this study. Following overnight food deprivation, anaesthesia was induced with 3% and maintained with 2.5% halothane in 2 l/min $O_2$. Via an abdominal incision, a SGT was sutured through each corner onto the extraluminal uterine wall. The recording lead was passed subcutaneously to emerge at a plug between the hen's shoulders. During initial operations it proved difficult to identify the uterine portion of the oviduct in the absence of an egg and so subsequent operations were performed in the morning when hens were carrying hard-shelled eggs. These eggs were either released during the operations or laid a few hours after. The position of the SGT on the oviduct was confirmed by post-mortem examination.

The SGTs were provided by G.E.Duke (University of Minnesota, USA). They were 14 x 10 x 2.5 mm, and were embedded in a silastic envelope with wires in 1.5 mm diameter silastic tubing. Their construction was described by Bass and Wiley (1972) and their use for measuring gut motility in birds by Duke and Kostuch (1975) and Savory *et al.* (1981). Flexing SGTs alters their electrical resistance and so they record changes in muscle shape and not changes in muscle tension.

Uterine contractions were recorded via a lead connecting the plug in the hen's back to a paper recorder (Elcomatic) with conditioning amplifiers (Fylde), a procedure that did not inhibit the hens' movements. During recording, hens were continuously observed to ensure that the lead did not become detached or trapped. For each recording trace the number of uterine contractions in each 2 min period was counted manually. Examples of these traces can be seen in Figure 5.1. In some recordings peaks appeared to have two components. It was assumed that these components reflected the contractions of longitudinal and circular muscles and so each double peak was counted as a single contraction.

Uterine contractions associated with normal oviposition were recorded from

hens in their home cages. Delayed oviposition was induced by relocation to a circular plastic cage (40 cm in diameter, 43-51 cm high) in an unfamiliar room. This stressor was used as exposure to unfamiliar hens, social stress, might disrupt the direct lead recording procedure. Uterine contractions associated with oviposition prematurely induced by 0.5 μg/kg PGF2α (as described in Section 6.2) were also recorded.

### 5.2.2 Results

Due to problems with SGT wires snapping, information about uterine contractions associated with oviposition was obtained from only two hens (#538 and #555). For some recordings there was much background "noise", presumably due to respiration and movements of other organs close to the SGT, and this made identification of uterine contractions more difficult. Typical recordings of uterine contractions 20 min before and immediately before normal oviposition are shown in Figure 5.1.

Figure 5.2 shows the uterine contraction frequencies associated with one normal oviposition of hen #538. Figure 5.3 shows the uterine contraction frequencies associated with three normal ovipositions and one oviposition induced prematurely by PGF2α injection for hen #555. A two-fold rise in uterine contraction frequency, from 20-25 in 2 min to 40-50 in 2 min, starting approximately 3 min before the egg was expelled was associated with each of these ovipositions. Once the egg was expelled the amplitude of the SGT recordings became very small and it was usually not possible to identify further contractions.

Uterine contraction frequencies associated with relocation to an unfamiliar environment and the resulting oviposition delay (47 min) are shown for hen #555 in Figure 5.4. There was no apparent effect of relocation on contraction frequency before oviposition. Figure 5.4 does not indicate any elevation of contraction frequency, relative to basal levels, at expected oviposition time or during the oviposition delay. Uterine contraction frequency rose about two-fold in association with the delayed oviposition and this was similar to the change in frequency associated with normal oviposition.

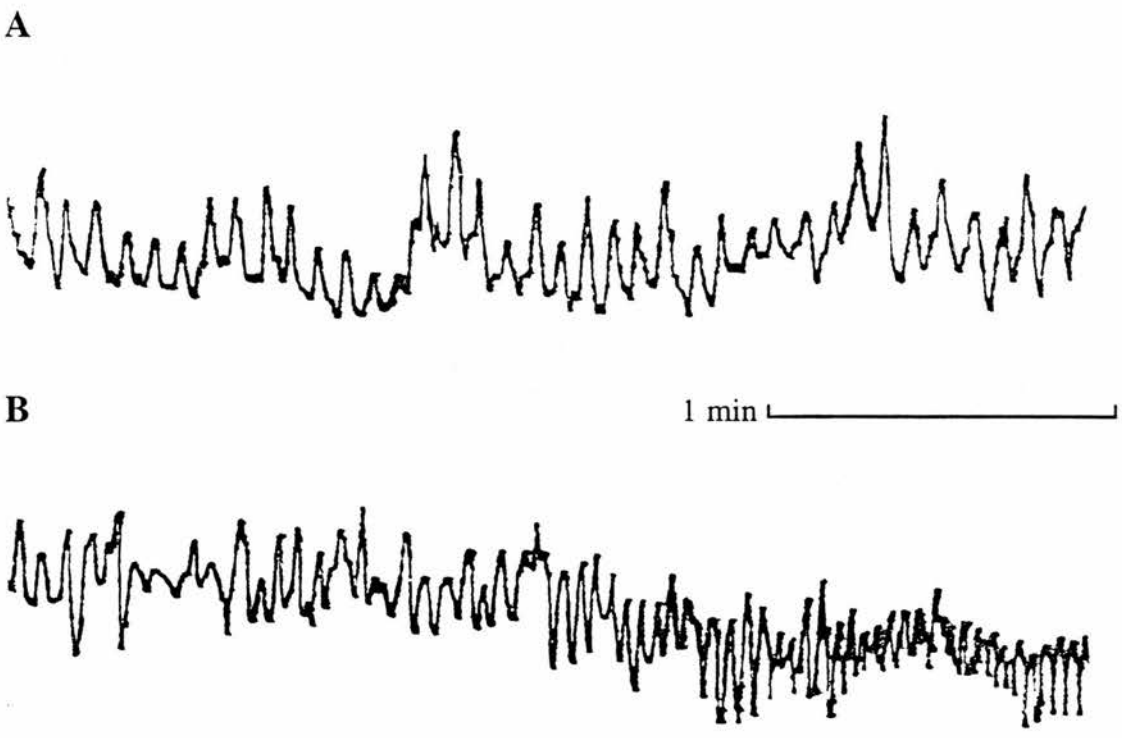



**Figure 5.1** Typical SGT recordings of uterine contractions 20 min before normal oviposition (A) and immediately before normal oviposition (B).

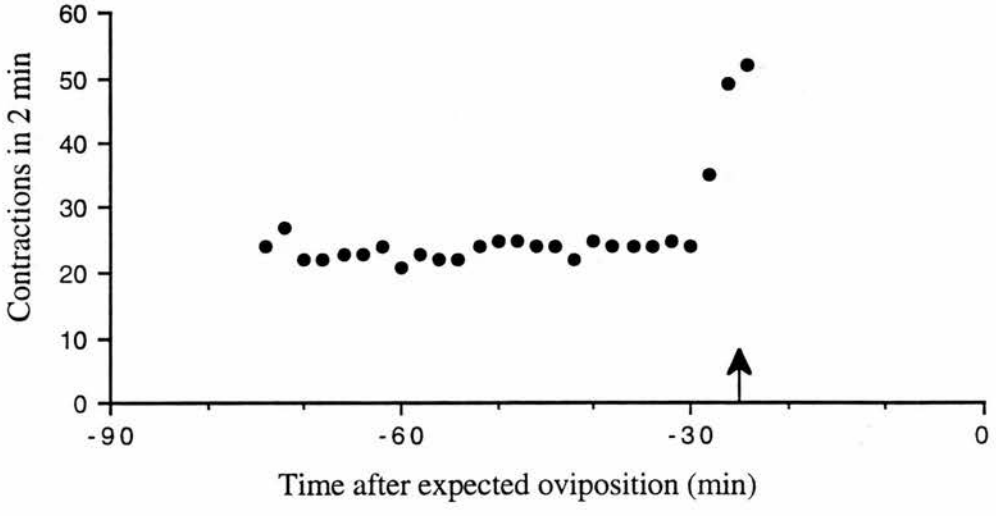



**Figure 5.2** Uterine contraction frequencies of hen #538 associated with one normal oviposition. Solid arrow indicates observed oviposition time. On the horizontal axis, negative values indicate frequencies recorded before expected oviposition time.

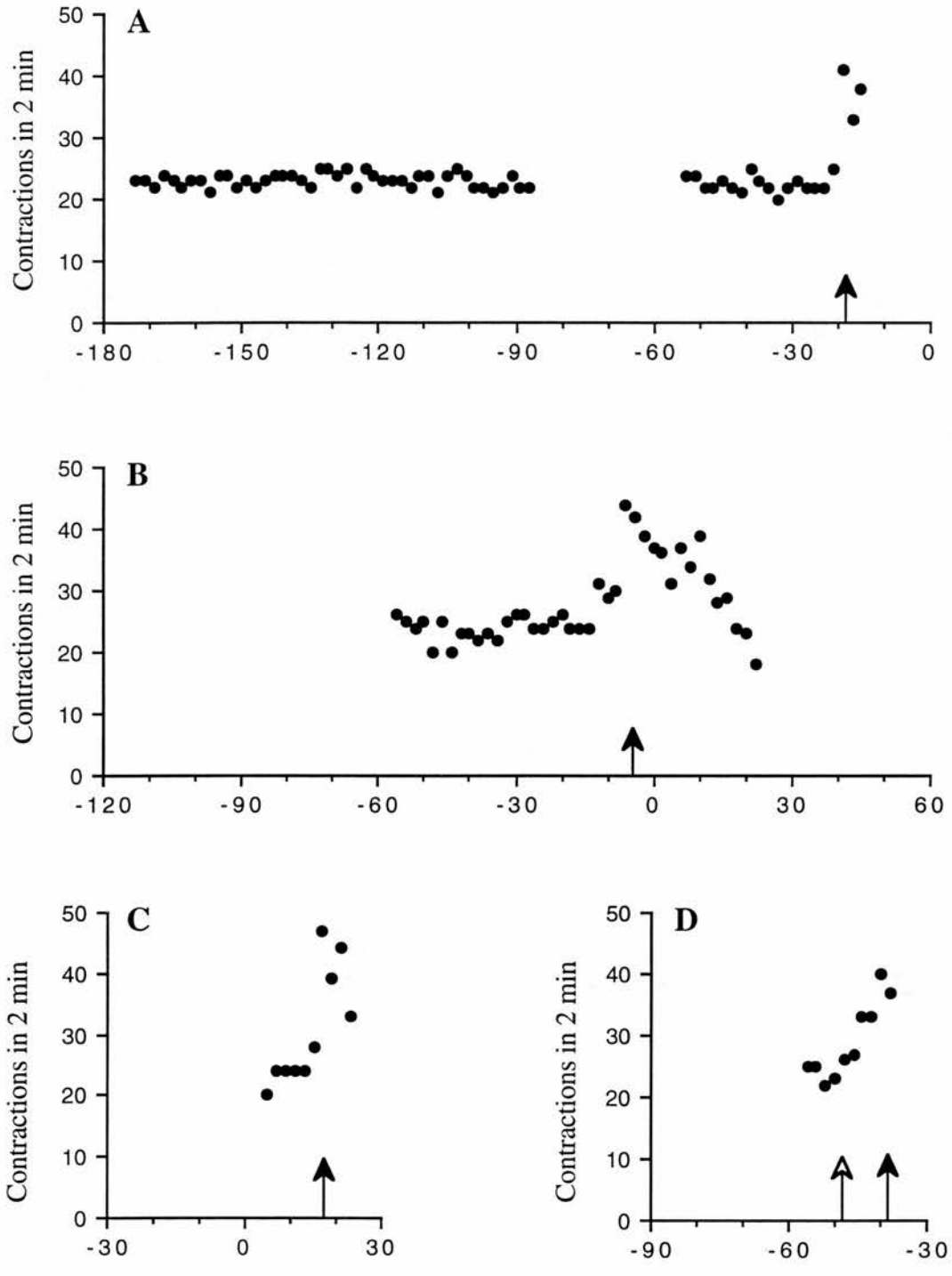



**Figure 5.3** Uterine contraction frequencies of hen #555 associated with three normal ovipositions (A, B and C) and one oviposition induced prematurely by 0.5 μg/kg PGF2α (D). As in Figure 5.2, horizontal axes show time after expected oviposition time. Solid arrows indicate observed oviposition time. Hollow arrow indicates PGF2α injection.

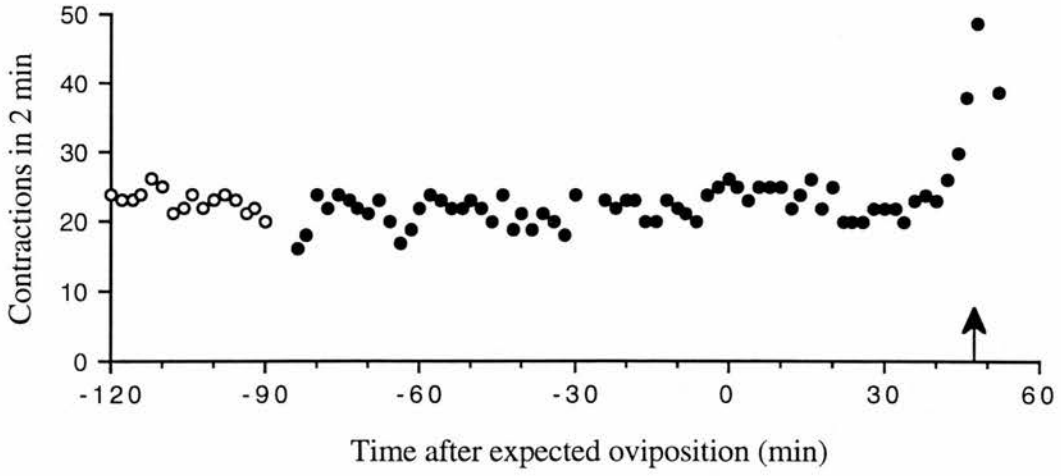



**Figure 5.4** Uterine contraction frequencies of hen #555 associated with one oviposition delayed by relocation to an unfamiliar environment. Hollow circles indicate contraction frequencies before relocation and solid circles indicate contraction frequencies after relocation. As in Figure 5.2, negative values on the horizontal axis indicate frequencies recorded before expected oviposition time.

## 5.4 Discussion

A two-fold rise in uterine contraction frequency from about 10 to 20 per min, starting about 3 min before the egg was expelled, was recorded in association with normal ovipositions. This change in frequency, measured by SGT, is the same as that measured previously by EMG (Shimada & Asai, 1978; Wechsung & Houvenaghel, 1988). A similar change in contraction frequency accompanied oviposition induced prematurely by PGF2α injection and this is also consistent with published reports (Shimada & Asai, 1979). These similarities between the present study, using SGT, and published studies, using EMG, suggest that both are recording the same events and so validate the use of SGTs for recording uterine contractions.

The change in uterine contraction frequency associated with an oviposition occurring after a short delay (47 min) was similar to that associated with normal ovipositions, and there was no evidence of increased frequency at expected oviposition time during the delay. One hypothesis to explain stress-induced oviposition delays is that they are due to inhibition of AVT release. If this hypothesis

is correct then an increase in uterine contraction frequency induced by ovarian PGs, the stimulus for AVT release (Shimada & Saito, 1989), should occur at expected oviposition time. There are two possible reasons why no such increase was found in the present study. First, contraction frequency was elevated during the oviposition delay (in a way that would normally initiate oviposition) but this increase was not detected because it was either too small in magnitude or too short in duration. Thus, the hypothesis that oviposition is delayed by inhibition of AVT release may be correct but the experimental technique used here was not sensitive enough to detect the rise in contraction frequency that stimulates AVT release. Second, an increase in contraction frequency did not occur and so the hypothesis is incorrect. Instead, oviposition is delayed because the stimulus for AVT release does not occur. Perhaps stress delays oviposition by preventing ovarian PGs from inducing uterine contractions, possibly by a direct adrenergic suppression of these muscles, or by inhibiting PG release. As neither the magnitude nor duration of the increase in uterine contraction frequency required to trigger AVT release have been described (see Introduction), it is not possible to differentiate between these two interpretations. Furthermore, only one delayed oviposition was recorded here and further investigation will be required before firm conclusions can be drawn.

One way in which the stimulus required for AVT release could be described is to measure uterine contraction frequencies associated with injections of exogenous PGs. By injecting doses ranging from those too low to cause oviposition to those which do cause oviposition, it might be possible to describe the maximum increase in uterine contraction frequency which does not initiate oviposition. This would suggest the minimum stimulus required to induce oviposition and presumably AVT release.

A second aim of this study was to investigate the suitability of SGTs for recording avian uterine contractions. Although changes in contraction frequency recorded in association with four normal ovipositions and one induced prematurely by PGF2$\alpha$ injection were similar to published reports, there were several factors which may limit the usefulness of SGTs for this application. Firstly, the amplitude of background "noise" on SGT recordings was often relatively high compared with the amplitude of uterine contractions, making some contractions difficult to identify. The presence of an incompressible egg presumably prevents the uterine muscles shortening and so uterine contractions will tend to cause changes in muscle tension (isometric contractions) rather than changes in muscle length (isotonic contractions). SGTs record changes in muscle shape and changes in muscle shape may be relatively small for isotonic contractions. Consequently, (unlike contractions in parts of the avian

gut), uterine contractions may cause only small changes in SGT shape and so deflections caused by respiration and movements of adjacent organs appear relatively large. Secondly, contractions of the empty uterus could not usually be recorded, yet EMG studies suggest that contraction frequency remains elevated for a short time after oviposition (Shimada & Asai, 1978; Wechsung & Houvenaghel, 1988). Presumably the empty uterus provides insufficient tension to flex SGT. Thus, although the problems encountered with wires snapping could presumably be overcome, there are other unresolvable problems which restrict the usefulness of SGTs for recording uterine contractions. Direct lead recording was also a limitation as this prevented the use of social stress.

In conclusion, SGTs are not ideally suited for measuring uterine contractions in hens, primarily because the egg restricts changes in muscle length. From recordings of uterine contraction frequency associated with one delayed oviposition, there was no evidence to suggest that contraction frequency is elevated during stress-induced oviposition delays. This may be because elevated contraction frequency, the stimulus for AVT release, did not occur, in which case the delays may not be due to inhibition of AVT release. Alternatively, a rise may have occurred but was too short or small to be detected.

# CHAPTER 6: **Oviposition Inducing Potency of Exogenous AVT and PGF2α in Relation to Stress and Oviposition Delays**

## 6.1 Introduction

Oviposition is initiated by the release of PGs - primarily PGF2α - from ovarian follicles (see Shimada & Saito (1989) for a review). These PGs increase the contraction frequency of uterine muscles and so trigger release of AVT from the neurohypophysis and into the circulation. This AVT further promotes uterine contractions, both directly and by stimulating production of PGs within uterine muscles, and so contributes towards expelling the egg from the uterus. Various environmental stressors induce oviposition delays (e.g. Hughes *et al.*, 1986) and one hypothesis is that these are due to opioidergic inhibition of AVT release (see Section 1.5) - a mechanism like that underlying stress-induced disruptions of parturition and lactation in mammals (e.g. Haldar & Bade, 1981; Petherick *et al.*, 1993). An alternative hypothesis is that stress might some how delay oviposition by inhibiting ovarian PG release (see Section 1.5).

The experiments described in Chapter 3 suggest that a threshold of about 3 h oviposition delay defines an upper limit beyond which oviposition of the delayed egg can no longer occur until the subsequent egg joins it in the uterus several hours later. Before this threshold (i.e. when oviposition delay has not yet reached about 3 h) ovipositions occurred shortly after (about 15 min) stress ending and often occurred during stress. These delays were described as short-term. However, once oviposition delays exceeded the 3 h threshold, ovipositions were unlikely to occur either during stress or if stress ended. Instead the eggs were laid much later, 7-15 h after expected oviposition time, and these delays were described as long-term. Thus, the threshold is defined as the time after expected oviposition time beyond which oviposition does not occur until much later (after a long-term delay). Several mechanisms to explain the threshold were hypothesised (see Section 3.4). Briefly the threshold may reflect changes in either release of, uterine responses to or half-lives of AVT and/or PGs.

Exogenous PGs can induce oviposition prematurely, an i.v. dose of 1 μg PGF2α/hen expelling eggs in a mean time of 7.8 min when injected 1-4 h before expected oviposition time (Goto *et al.*, 1985). Assuming each hen in that study weighed around 2 kg, 1 μg/hen would be about 0.5 μg/kg. Murakami *et al.* (1990)

found that plasma AVT concentrations rise in association with ovipositions induced prematurely by intra-uterine injection of 1 μg PGF2α/hen. They suggest that neurohypophysial AVT is released in response to an increase in uterine contraction frequency caused by the PGF2α and this AVT acts to further promote uterine contractions and so to assist with expulsion of the egg. Exogenous AVT also induces premature oviposition, an i.v. dose of 0.1 μg/kg expelling eggs in a mean time of 5.4 min when injected 2-3 h before expected oviposition time (Rzasa & Ewy, 1970). Ovarian PGs are not released when oviposition is induced prematurely by AVT (Olson & Hertelendy, 1981).

To investigate the mechanisms underlying stress-induced oviposition delays and the threshold (beyond which delayed ovipositions do not occur until much later; see above), plasma AVT and PGF2α concentrations were elevated by injection in the following experiments. If stress induces oviposition delays by a mechanism other than inhibition of ovarian PG release (e.g. by inhibiting AVT release or suppressing uterine contractions), and if such a mechanism is also active when hens are exposed to stress before expected oviposition time, then stress might reduce the ability of exogenous PGF2α to induce premature oviposition. Experiment 1 tested this possibility. If oviposition delays are due to inhibition of neurohypophysial AVT release then injection of exogenous AVT should end these delays. Alternatively, if oviposition delays are due to inhibition of ovarian PG release then injection of exogenous PG should end these delays. Experiment 2 investigated the effects of AVT injections and of PGF2α injections on stress-induced oviposition delays. If the threshold is due to either changes in uterine response to AVT and/or PGF2α or changes in the half-lives of these hormones then the ability of AVT and/or PGF2α injections to end delays is likely to be reduced after the threshold. Experiment 3 investigated the abilities of exogenous AVT and PGF2α to end long-term stress-induced oviposition delays.

## 6.2 Does Stress Affect Premature Induction of Oviposition by Exogenous PGF2α?

The aim of this experiment was to investigate the effect of social stress on induction of premature oviposition by PGF2α injection.

### 6.2.1 Materials and Methods

From 35 stock hens aged 57 weeks, 21 whose next oviposition times could be predicted were selected and randomly allocated to receive a PGF2α injection following either no disturbance (control, n = 11) or a period of social stress which started approximately 3 h before predicted oviposition time (stress, n = 10). A stock solution of 1 mg PGF2α (PGF2α tris salt, Sigma) in 1 ml of 95% ethanol was prepared and stored at -20°C. Each day, 25 µl of stock PGF2α was diluted with 0.9% saline to 1 ug/ml. Injections were 0.5 ml/kg i.v. and approximately 1.5 h before predicted oviposition time (i.e. during short-term oviposition delays). This dose of PGF2α (0.5 µg/kg) is similar to the lowest with which Goto *et al.*, (1985) were able to induce oviposition 1-4 h prematurely. The times from injection until observed oviposition (oviposition induction times) were recorded. Control hens were replaced in their cages immediately after injection. As it was not known if PGF2α injection would make hens more vulnerable to injury from other birds by inducing oviposition, hens exposed to social stress were not returned to group cages after injection. Instead, they were placed in an open cardboard box (base = 25 cm x 45 cm) in an unfamiliar room and this was intended to maintain stress in the form of an unfamiliar environment.

### 6.2.2 Results

For 7 hens (4 x stress treatment, 3 x control treatment), injection was within 2 days of the egg sequence ending and, as this prevented accurate calculation of expected oviposition time, these hens were excluded from the analysis. Another hen (control treatment) received its injection sub-cutaneous rather than i.v. and was also excluded from the analysis.

Injections were 57-186 min (mean = 108 min) before expected oviposition time. Ovipositions occurring within 15 min of injection were considered to have been induced. Effects of control and stress treatments on induction of premature oviposition by PGF2α are shown in Table 6.1. Oviposition induction times were compared using rank statistics, with the highest rank allocated to the two non-induced

ovipositions. There was no difference in oviposition induction times between stress and control treatment (Mann-Whitney, w=30.5, $P>0.05$).

| | Control | Stress |
|---|---|---|
| Number of injected hens | 7 | 6 |
| Number of ovipositions induced | 6 | 5 |
| Mean oviposition induction time (min) | 3.2 | 2.3 |
| | (0.5) | (0.1) |

**Table 6.1** Effects of control and stress treatments on the number of ovipositions induced prematurely by 0.5 μg/kg PGF2α and on mean (±SEM) oviposition induction times.

## 6.3 Do Injections of AVT or PGF2α End Short-Term Stress-Induced Oviposition Delays?

The aim of this experiment was to find out if short-term stress-induced oviposition delays could be ended by injection of PGF2α or AVT.

### 6.3.1 Materials and Methods

From 58 stock hens aged 32 weeks, 27 whose next oviposition times could be predicted were selected. To induce oviposition delays, selected hens were each exposed to social stress, starting 30 min before predicted oviposition time, on one of three days (n = 9 each day). Each day, hens were randomly allocated to receive an injection of either 0.9% saline, 0.1 μg/kg AVT or 0.5 μg/kg PGF2α (n = 3 each) during their oviposition delays. Each day, 27 unselected stock hens were randomly divided into 9 groups of 3 in such a way that hens in each group were strangers to each other. Each of the 9 selected hens was exposed to social stress by housing it with one of these groups.

AVT ([ARG$^8$]-vasotocin acetate salt, Sigma) was dissolved in 0.9% saline (0.2 μg/ml) and stored at -18$^o$C until required. Preparation of PGF2α is described in Section 6.2.1. All injections were 0.5 ml/kg i.v. and 40 min after predicted

oviposition time. Thus, injections were after oviposition had been delayed for 40 min and were 70 min after stress started. This dose of AVT (0.1 μg/kg) induces oviposition 2-3 h prematurely (Rzasa & Ewy, 1970). From hens' responses to PGF2α injection in Experiment 1, it was decided that hens should be returned to their group cages immediately after injection in the present experiment. Oviposition induction times were recorded. All hens in a group were returned to their home cages as soon as the injected hen laid or, if this did not occur, 3 h after its predicted oviposition time.

### 6.3.2 Results

Seven hens (2 x AVT treatment, 5 x PGF2α treatment) laid during stress and < 40 min after predicted oviposition time. These hens were not injected as they no longer carried eggs at 40 min after predicted oviposition time (the intended injection time).

For the remaining hens, oviposition delays due to social stress were 14-61 min (mean = 42 min) at the time of injection. Four hens receiving saline did not lay during stress and so their oviposition induction times were calculated as the time from injection until 3 h past expected oviposition time. All AVT and PGF2α injections induced oviposition within 10 min (Table 6.2). As some saline treated hens did not lay during stress, oviposition induction times were compared between pairs of treatments using rank statistics. Both AVT and PGF2α injections induced oviposition more rapidly than did saline injections (respectively by Mann-Whitney: w=28, $p<0.001$; w=10, $P<0.01$). Thus, the oviposition delaying effect of social stress was ended by injection of either 0.1 μg/kg AVT or 0.5 μg/kg PGF2α.

| Injection | n | Mean oviposition induction time (min) |
|---|---|---|
| Saline | 9 | 102.3 (17.6) |
| AVT | 7 | 2.0 (0.5) |
| PGF2α | 4 | 5.1 (1.7) |

**Table 6.2** Mean (±SEM) oviposition induction times following injections of either saline, 0.1 μg/kg AVT or 0.5 μg/kg PGF2α for hens delaying oviposition short-term during social stress.

## 6.4 Do Injections of AVT or PGF2α End Long-Term Stress-Induced Oviposition Delays?

The aim of this experiment was to find out if injections of AVT or PGF2α would end long-term stress-induced oviposition delays. Only about one quarter of hens exposed to social stress can be expected to delay oviposition beyond the threshold (see Figures 3.1 and 3.4 and Section 3.4) and this creates two problems when designing experiments to investigate its underlying mechanisms. First, to induce a required number of long-term oviposition delays for an experiment, about four times as many hens must be exposed to social stress. Second, it cannot be assumed that hens delaying oviposition beyond the threshold represent a random sample of the whole population as some hens may be more likely than others to delay oviposition for this long. It is not possible to predict which hens will delay their ovipositions beyond the threshold. Therefore, a large and complex experiment would be required to compare the effects of AVT and PGF2α injections on long-term oviposition delays with similar injections at other times (e.g. before expected oviposition time and during short-term oviposition delay). As such an experiment was impractical, the effects of these injections on long-term oviposition delays were not directly compared with their effects at other times and this experiment was undertaken as an exploratory study.

### 6.4.1 Materials and Methods

*AVT injections*

Eighteen hens aged 48 weeks were each exposed to social stress for 4 h, starting 0.5 h before predicted oviposition time. From Chapter 3, this treatment was expected to induce long-term oviposition delays (i.e. delays which do not end before the threshold and are therefore 7-15 h in duration) in about 1/4 of the hens. Four hens did not lay during exposure to stress or in the hour after they were returned to their original cages, and it was assumed that their ovipositions were delayed long-term. These 4 hens each received 0.1 μg/kg AVT (see Section 6.3.1) approximately 6 h after predicted oviposition time and oviposition induction time was recorded. As long-term oviposition delays cause white-banded and slab-sided eggshells (see Chapter 3), eggshell abnormalities were also recorded.

*PGF2α injections*

Eighteen hens aged 21 weeks were exposed to social stress as described above. Four hens delayed oviposition long-term and each received 0.5 μg/kg PGF2α (see Section 6.2.1) approximately 5 h after predicted oviposition time. Oviposition induction times and eggshell abnormalities were recorded.

### 6.4.2 Results

*AVT injections*

For each hen, oviposition delay at time of injection and oviposition induction time are shown in Table 6.3. Both eggs not laid within 15 min of injection were white-banded when they were eventually laid, at an unknown time, and the egg following each was slab-sided. Although both eggs laid within 15 min of injection appeared normal, the egg following each was soft-shelled and was laid prematurely at an unknown time.

| Oviposition delay at time of AVT injection (h) | Oviposition induction time (min) |
|---|---|
| 6.5 | 3.5 |
| 5.2 | 3.0 |
| 6.2 | >15.0 |
| 6.3 | >15.0 |

**Table 6.3** Oviposition induction time following AVT injection (0.1 μg/kg) for each of 4 hens delaying oviposition long-term in response to social stress.

*PGF2α injections*

For each hen, oviposition delay at time of injection oviposition and induction time are shown in Table 6.4. The three eggs not laid within 15 min of injection were white-banded when they were eventually laid, at an unknown time, and the egg following each was slab-sided. Neither the egg laid within 15 min of injection nor the egg following it appeared abnormal.

| Oviposition delay at time of PGF2α injection (h) | Oviposition induction time (min) |
|---|---|
| 6.6 | >15.0 |
| 4.6 | >15.0 |
| 4.1 | >15.0 |
| 5.0 | 11.6 |

**Table 6.4** Oviposition induction time following PGF2α injection (0.5 μg/kg) for each of 4 hens delaying oviposition long-term in response to social stress.

## 6.5 Discussion

In Section 6.2, there was no difference between stress and control treatments in the time taken for exogenous PGF2α to induce premature oviposition. If stress causes oviposition delays by disrupting the oviposition process subsequent to ovarian PG release, e.g. by inhibiting AVT release or by directly suppressing uterine contractions, then it might be expected to increase the time taken for PGF2α to induce oviposition. However this did not occur, so the results are consistent with the hypothesis that oviposition delays are caused by inhibition of ovarian PG release. However, as discussed below, a dose of 0.5 μg/kg PGF2α may be sufficient to overcome other mechanisms which disrupt oviposition. Another two explanations may account for the similar effects of stress and control treatments. First, the injection procedure was itself stressful and so hens from both treatments may have been stressed when injected. Second, hens from the stress treatment were isolated in a box after injection and this situation may have been less stressful than the group cage, so reducing any effect of social stress on premature induction of oviposition.

In Section 6.3, short-term oviposition delays were ended by exogenous AVT or PGF2α. Interpretation of this, with respect to the mechanisms underlying stress-induced oviposition delays, is complicated by the fact that elevated plasma PG levels cause AVT release (by inducing uterine contractions) and therefore AVT release is not independent of elevated plasma PG (exogenous or endogenous).

The finding that injections of exogenous AVT end short-term stress-induced oviposition delays is considered first. This is consistent with the hypothesis that these delays are due to inhibition of AVT release. In mammals, stress-induced disruptions of parturition and lactation are at least partly due to inhibition of oxytocin release by

endogenous opioids (e.g. Haldar & Bade, 1981; Petherick *et al.*, 1993), and hence opioids might act to inhibit AVT release in hens. However, as AVT injections induce premature oviposition without causing release of ovarian PGs (Olson & Hertelendy, 1981), the finding here that AVT injections end stress-induced oviposition delays is also consistent with the alternative hypothesis that these delays are due to inhibition of the stimulus for AVT release, i.e. ovarian PG release.

The finding that injections of exogenous PGF2α end short-term stress-induced oviposition delays is also consistent with the hypothesis that delays are due to inhibition of ovarian PG release. Little is known about the mechanisms controlling ovarian PG release (Shimada & Saito, 1989), and it is not clear how they might be disrupted by stress. AVT is released in association with oviposition induced prematurely by exogenous PGF2α and is thought to contribute towards expelling the egg (Murakami *et al.*, 1990). If the effects of AVT are important for the induction of oviposition by PGF2α injection, and if AVT release is inhibited by the stress causing oviposition delays, then it might be expected that PGF2α injections would end these delays relatively slowly or not at all. However, exogenous PGF2α ended all four oviposition delays and, although not directly comparable, the mean induction time of 5.1 min is similar to that (7.8 min) following 1 μg/hen PGF2α i.v. 1-4 h before expected oviposition time (Goto *et al.*, 1985). Hence, AVT release following PGF2α injection is either not inhibited by social stress or not essential for induction of oviposition.

If a mechanism other than inhibition of AVT or PG release causes stress-induced oviposition delays, then injection of either 0.1 μg/kg AVT or 0.5 μg/kg PGF2α is clearly able to overcome it. If the plasma volume of hens is assumed to be 6% of their body weight (Medway & Kare, 1959), injecting 0.1 μg/kg AVT should raise plasma concentration by 1670 pg/ml. The increase in plasma AVT associated with normal oviposition is much less than this at around 60 pg/ml (Tanaka *et al.*, 1984). Injection of 0.5 μg/kg PGF2α should elevate plasma concentration by 8330 pg/ml. Measurements of the rise in plasma PGF2α at normal oviposition vary, including rises of between 500 and 1000 pg/ml and less than 100 pg/ml (from the figures of Olson *et al.* (1986) and Saito *et al.* (1987) respectively). Thus, plasma AVT and PGF2α concentrations following these injections will have been considerably higher than those associated with spontaneous oviposition and this must be considered when interpreting the results. Perhaps stress-induced oviposition delays are due to inhibition of AVT release, but the dose of exogenous PGF2α was sufficient to end these delays despite reduced AVT. Alternatively, adrenaline released in response to stress may delay oviposition by directly suppressing uterine contractions (Weiss &

Sturkie, 1952; Hughes & Black, 1976; Crossley, 1983). The doses of AVT and PGF2α injected might overcome adrenergic suppression of uterine contractions to end oviposition delays. Thus, although short-term stress-induced oviposition delays were ended by AVT or PGF2α injection, it cannot be concluded that the delays were due to inhibition of these hormones as the doses administered may have been sufficient to override other mechanisms.

In Section 6.4, injections of AVT or PGF2α were inconsistent at inducing oviposition of eggs delayed by social stress beyond the 3 h threshold. Equivalent injections rapidly induce premature oviposition (Rzasa & Ewy, 1970; Goto *et al.*, 1985; also see Section 6.2) and end short-term oviposition delays (Section 6.3). This supports the hypotheses that oviposition does not occur after the threshold because either uterine sensitivity to AVT or PGs is reduced or the half-life of these hormones in circulation is reduced (see Chapter 3). A reduction in sensitivity to AVT or PGs could be due to changes in numbers of specific receptors and changes in the binding characteristics of these, while changes in circulating levels of appropriate enzymes could affect the half-lives of these hormones (see p. 44 & 45). For a further discussion of mechanisms responsible for the threshold, see Chapter 12.

Exogenous AVT and PGs induce uterine contractions and oviposition most potently when injected shortly before expected oviposition time, and become progressively less potent if they are injected at earlier times in the egg cycle (Rzasa & Ewy, 1970; Goto *et al.*, 1985; Shimada *et al.*, 1987). However, as there is not normally an egg in the uterus 6 h after oviposition, the effects of AVT and PG injections on uteri containing eggs at this time have not previously been reported. In the present study (Section 6.4) stress was used to delay oviposition so that there was an egg in the uterus 6 h after normal (expected) oviposition time. At this time, injection of either AVT or PGF2α induced oviposition in only a small proportion of hens and then only relatively slowly (compared with Section 6.3). This suggests that the uterine contraction inducing potency of these injections may be low in the period after normal oviposition time and before the next egg enters the uterus.

Long-term oviposition delays not ended by AVT or PGF2α injection resulted in white-banded and slab-sided eggs and this is consistent with the findings of Chapter 3 and those of van Middelkoop (1971) and Hughes *et al.* (1986). It is not clear why eggs subsequent to those expelled by AVT during long-term delays were laid prematurely with soft shells. An intra-uterine irritant in the form of a loop of thread sutured to uterine muscle causes premature oviposition (Sykes, 1953b; Lake & Gilbert, 1964; Ogasawara *et al.*, 1974). Perhaps expulsion of long-term delayed eggs by AVT causes damage or irritation to the uterus and this causes premature expulsion

of the next egg. However, premature oviposition of the next egg did not occur when a long-term delay was ended by PGF2α injection.

To summarise, there was no effect of stress on induction of premature oviposition by exogenous PGF2α. Injections of AVT or PGF2α rapidly ended short-term stress-induced oviposition delays but only ended a small proportion of long-term oviposition delays and then only relatively slowly.

# CHAPTER 7: **Do Opioid Antagonists Reduce the Duration of Stress-Induced Oviposition Delays?**

## 7.1 Introduction

It is hypothesised that opioids released in response to stress cause oviposition delays in hens by inhibiting neurohypophysial AVT release (see Section 1.6). In mammals, stress-related disruptions of parturition and lactation are due to a similar opioidergic inhibition of oxytocin release (see Petherick *et al.* (1993) for a review). The ability of opioid receptor antagonists to reduce these disruptions implicates endogenous opioids in the underlying mechanisms. For example, moving rats from their nesting cages to the unfamiliar environment of a glass chamber mid-parturition prolongs labour, a response which is reduced by treatment with naloxone, a non-specific opioid receptor antagonist, (Leng *et al.*, 1987; Leng *et al.*, 1988). Naloxone also reduces the disruption of parturition caused by moving sows to an unfamiliar environment after the birth of their first piglet (Lawrence *et al.*, 1992). Naltrexone, another non-specific antagonist, reduces the inhibition of milk yield induced by briefly relocating lactating mice to an unfamiliar and hot environment (Haldar & Bade, 1981).

The experiments reported in this chapter investigated the role of endogenous opioids in the mechanisms underlying stress-induced oviposition delays through treatments with three opioid antagonists: nalmefene, MR2266 and nor-binaltorphimine (norBNI). If opioids are involved in the causation of oviposition delays then blocking the appropriate receptors with an antagonist should reduce the duration of these delays.

Nalmefene is a non-specific opioid antagonist which increases headflick frequency and suppresses feeding, drinking and stereotyped pecking in fowls (Savory & Hughes, 1988; Savory *et al.*, 1989; Savory *et al.*, 1992). MR2266, which has highest affinity for kappa opioid receptors but is also potent at mu receptors, also partially suppressess stereotyped pecking in broiler breeder fowls (Kostal & Savory, 1994). NorBNI is a more specific antagonist of kappa receptors than is MR2266, although it crosses the blood brain barrier relatively poorly (McKnight & Rees, 1991). NorBNI has been used as a specific kappa receptor antagonist in studies of memory formation in chicks (Colombo *et al.*, 1992) and investigations into the effects of kappa agonists on isolated chick neurone membranes (Fletcher & Chiappinelli, 1993; Maderspach & Nemeth, 1993). Further details concerning the selectivity of these antagonists are given in Table 1.3.

As naltrexone and naloxone are the most commonly used non-specific opioid receptor antagonists in mammalian studies it is appropriate to compare their pharmacological properties with those of nalmefene. The affinity of nalmefene for mu receptors is similar to that of naltrexone but about four times greater than that of naloxone (Michel *et al.*, 1985). Its affinity for kappa and delta receptors is 2-3 times greater than that of naltrexone and 4-12 times greater than that of naloxone (Michel *et al.*, 1985). In behavioural tests, nalmefene antagonises morphine induced analgesia much more potently than either naltrexone or naloxone (Hahn *et al.*, 1975). In addition to its greater potency, nalmefene antagonises opioid receptors for longer durations than either naltrexone or naloxone (Vizi *et al.*, 1976; VanVugt, 1989).

Delta opioid receptors have not been implicated in the control of oxytocin release in mammals (Bicknell *et al.*, 1985) and so the effects of specific delta opioid receptor antagonism was not investigated in the present study. Although naloxone and naltrexone are often used as mu-receptor antagonists they are not very specific and no truly selective non-peptide blocker is available (McKnight & Rees, 1991). Consequently, the effect of selective mu-receptor antagonism on the duration of stress-induced oviposition delays was not investigated.

## 7.2 Does Treatment with Nalmefene Reduce the Duration of Stress-Induced Oviposition Delays? Experiment 1

The aim of this experiment was to investigate the effect of nalmefene on the duration of stress-induced oviposition delays.

### 7.2.1 Materials and Methods

From 36 naive stock hens aged 27 weeks, 18 whose next oviposition times could be predicted were selected over two days and randomly allocated to receive an injection of either 0.9% saline or nalmefene (n = 9 each), each followed by a period of social stress. Nalmefene (6-desoxy-6-methylene naltrexone; Key Pharmaceuticals, Miami) was dissolved in 0.9% saline (1 mg/ml) and stored at -18$^{o}$C until required. Nalmefene and saline were both injected i.v. at 0.5 ml/kg, 30 min before predicted oviposition time. This dose of nalmefene was expected to provide a high level of opioid antagonism without sedation (Savory & Hughes, 1988).

Immediately after injection, each hen was exposed to stress by placing it in a group cage containing five unfamiliar hens. This stress was ended by returning the

hen to its home cage once it had laid, or after a period of 3.5 h if oviposition did not occur. As there were insufficient unselected stock hens to make up independent stress groups for each of the 18 injected hens, some group cages contained more than one injected hen. As predicted oviposition times varied, periods of stress began at different times for each hen and so individual injected hens were never exposed to each other for the full duration of their stress periods. When injected hens were added to or removed from group cages, the number of stock birds was altered to maintain the group size of six.

As individual treated hens were not exposed to each other for the full duration of their stress period, group effects were not included in the analysis. It was to reduce the influence of individual hens on the level of stress experienced by others in the same group that a group size of six was used here, rather than four as in previous experiments.

### 7.2.2 Results

One hen (saline treatment) did not lay during exposure to stress and its oviposition delay was considered to be 180 min for the following analysis. Table 7.1 shows the mean durations of stress-induced oviposition delay after injection of either saline or nalmefene. Delays were significantly longer after saline injection than after nalmefene injection (Mann-Whitney; w=55, $P<0.01$).

| Injection | Mean duration of oviposition delay (min) | |
|---|---|---|
| Saline | 82.3 | (15.4) |
| Nalmefene | 17.2 | (11.4) |

**Table 7.1** Mean (±SEM) durations of oviposition delay for hens exposed to social stress after injection of either saline or 0.5 mg/kg nalmefene (n = 9 each) in Experiment 1.

## 7.3 Does Treatment with Nalmefene Reduce the Duration of Stress-Induced Oviposition Delays? Experiment 2

In Experiment 1, treatment with nalmefene reduced the duration of stress-induced oviposition delays. However, as group composition often changed during stress periods and some injected hens were placed in the same group cage, hens did not all receive identical and unrelated periods of stress. The aim of Experiment 2 was to confirm the results of Experiment 1 and to avoid the above criticisms through the use of an improved experimental design.

### 7.3.1 Materials and Methods

Twenty-seven Hisex hens aged 29 weeks were housed individually in bottom tier battery cages. These hens had been used in a study of feather pecking development (Savory & Mann, submitted) during which they were housed in floor pens in groups of 5-8 until 26 weeks of age, when they were transferred to the battery cages. An additional 69 stock hens (ISA Brown, 39 weeks old), housed individually in middle tier battery cages, were used to make up groups for stressing the injected hens.

From the 27 hens, 23 whose next oviposition times could be predicted were selected and randomly allocated to receive an injection of either 0.9% saline (n = 11) or 0.5 mg/kg nalmefene (n = 12), each followed by a period of social stress. These injections are described in Section 7.2. Immediately after injection, each hen was exposed to social stress by placing it in a group cage with three stock hens for a period of 4.0 h, after which it was returned to its original cage. Hence, in contrast to Experiment 1, the composition of each stress group was constant, all hens were exposed to stress for the same duration and all stress groups were independent.

### 7.3.2 Results

Two hens laid before they were due to be injected and another laid as it was restrained to receive its injection. These three hens (2 x nalmefene treatment, 1 x saline treatment) were excluded from the analysis (so, n = 10 for both treatments). Table 7.2 shows the mean durations of stress-induced oviposition delay following injection of either saline or nalmefene. Delays were significantly longer after saline injection than after nalmefene injection (Mann-Whitney; w=7.65, $P<0.05$).

| Injection | Mean duration of oviposition delay (min) |
|---|---|
| Saline | 29.0 (9.0) |
| Nalmefene | 5.5 (4.7) |

**Table 7.2** Mean (±SEM) durations of oviposition delay for hens exposed to social stress after injection of either saline or 0.5 mg/kg nalmefene (n = 10 each) in Experiment 2.

## 7.4 Does Treatment with MR2266 Reduce the Duration of Stress-Induced Oviposition Delays?

In Sections 7.2 and 7.3 the duration of stress-induced oviposition delays was reduced by treatment with the non-selective opioid receptor antagonist nalmefene, thus implicating endogenous opioids in the causation of these delays. The aim of the following experiment was to investigate the role of kappa opioid receptors through use of the preferential kappa receptor antagonist MR2266.

### 7.4.1 Materials and Methods

From 35 stock hens aged 29 weeks, 20 whose next oviposition times could be predicted were selected over three days and randomly allocated to receive an injection of either 0.9% saline or 2 mg/kg MR2266 (n = 10 each), each followed by a period of social stress. MR2266 (Boehringer, Ingelheim, Germany) was dissolved in 0.1M hydrochloric acid, then made up to a concentration of 2 mg/ml with 0.9% saline. Both the MR2266 solution and the saline for control injections were adjusted to pH 5.9 by titration with sodium hydroxide. Injections were i.v., 1 ml/kg and 30 min before predicted oviposition time. This dose of MR2266 corresponds to the highest dose used in a study of the role of opioid receptor subtypes in stereotyped behaviour of broiler breeder fowls (Kostal & Savory, 1994).

Immediately after injection, each hen was exposed to social-stress by placing it in a group cage containing seven unfamiliar hens. This stress was ended by returning the hen to its home cage once it had laid, or after a period of 3.5 h if oviposition did not occur. As there were insufficient remaining stock hens to make up independent stress groups for each of the 20 injected hens, some injected hens were stressed in the

same cage. Predicted oviposition times varied and so each hen's period of stress began at a different time. When an injected hen was added to or removed from a group cage, the number of stock hens was altered to maintain the group size of eight. As group composition varied during the stress periods, injected hens were not exposed to each other for the full duration of their stress periods and so group effects were not included in the analysis. A group size of eight was used here, rather than four or six as in previous experiments, in order to reduce the influence of individual hens on the level of stress experienced by others in the same group.

### 7.4.2 Results

Four hens (3 saline and 1 MR2266) did not lay during exposure to stress and their oviposition delays were considered to be 180 min for the following analysis. Table 7.3 shows the mean durations of stress-induced oviposition delay after injection of either saline or MR2266. As the data were normally distributed and had an even distribution of variance, parametric statistics (GLM) were used for the analysis. Neither treatment ($F=1.35$; 1, 14 df; $P>0.05$), day ($F=1.75$; 2, 14 df; $P>0.05$) nor an interaction between day and treatment ($F=1.04$; 2, 14 df; $P>0.05$) had a significant effect on the duration of oviposition delays.

| Injection | Mean duration of oviposition delay (min) | |
|---|---|---|
| Saline | 92.0 | (21.7) |
| MR2266 | 54.8 | (17.6) |

**Table 7.3** Mean (±SEM) durations of oviposition delay for hens exposed to social stress after injection of either saline or 2 mg/kg MR2266 (n = 10 each).

## 7.5 Does Treatment with nor-Binaltorphimine Reduce the Duration of Stress-Induced Oviposition Delays?

In Section 7.4, treatment with the kappa receptor antagonist MR2266 slightly, but not significantly, reduced the duration of stress-induced oviposition delays. Perhaps complete antagonism of kappa receptors was not achieved with 2 mg/kg MR2266 and a higher dose would have reduced delay duration more effectively. However, MR2266 antagonises mu receptors in addition to kappa receptors (Magnan *et al.*, 1982), and so the effect of MR2266 cannot be attributed unambiguously to kappa receptor antagonism. The aim of the following experiment was to investigate the effect of norBNI, a kappa opioid receptor antagonist with higher selectivity than MR2266, on the duration of stress-induced oviposition delays.

### 7.5.1 Materials and Methods

From 48 naive stock hens aged 25 weeks, 18 whose next oviposition times could be predicted were selected and randomly allocated to receive an injection of either 0.9% saline or 0.5 mg/kg norBNI, both followed by a period of social stress. As there were insufficient unselected stock hens to make up 18 independent stress groups, two selected hens and two stock hens were placed in each group cage (thus, there were four birds in each stress group). The 18 selected hens were divided into pairs with similar predicted oviposition times. One hen from each pair was randomly chosen to receive norBNI, with the other receiving saline.

NorBNI (17,17'-bis(cyclopropylmethyl)6,6',7,7'tetradehydro 4,5,4',5'-diepoxy-6,6'(imino)[7,7'-bimorphinan]-3,3',14,14'tetroldihydrochloride; Research Biochemicals Incorporated, Natick, MA) was dissolved in 0.9% saline (1 mg/ml). This solution was prepared on the day of injection as norBNI is unstable in solution. NorBNI and saline were both injected i.v. at 0.5 ml/kg, 30 min before predicted oviposition time. The dose of norBNI (0.5 mg/kg) was chosen as, when injected i.v., it fully prevents the disruption of rat parturition induced by U50,488 (Douglas *et al.*, 1993). Periods of social stress began immediately after injection and lasted 3.5 h.

### 7.5.2 Results

Table 7.4 shows the mean durations of stress-induced oviposition delay after injection of either saline or norBNI. Although delay duration was greater for hens

receiving norBNI than for hens receiving saline, this difference was not significant (paired t test: t=0.78, $P>0.05$).

| Injection | Mean duration of oviposition delay (min) | |
|---|---|---|
| Saline | 86.4 | (22.4) |
| norBNI | 108.9 | (24.8) |

**Table 7.4** Mean (+SEM) durations of oviposition delay for hens exposed to social stress after injection of either saline or 0.5 mg/kg norBNI (n = 9 each).

## 7.6 Discussion

In the present experiments social stress caused hens to delay oviposition and this is consistent with the findings reported in earlier chapters. The duration of these delays was significantly reduced by nalmefene and this suggests that endogenous opioids are involved in their underlying physiological causation. In mammals, environmental stressors disrupt the milk ejection reflex and prolong parturition, processes dependant on neurohypophysial oxytocin. These disruptions can be reduced by treatment with a non-specific opioid antagonist and it has been shown that they are due to an opioid mediated inhibition of oxytocin release (e.g. Haldar & Bade, 1981; Leng *et al.*, 1988). In hens, neurohypophysial AVT is released at oviposition and is involved in inducing the uterine contractions which expel the egg (Shimada & Saito, 1989). It is hypothesised that stress induced oviposition delays may be due to an opioidergic inhibition of AVT release (see Section 1.6). The finding that nalmefene reduces the duration of these delays supports this hypothesis. At the magnocellular neurone level, opioids can inhibit hormone release in mammals by acting either within the neurohypophysis (peripherally, kappa receptors) or within the hypothalamus (centrally, mu and kappa receptors) (see Bicknell (1985) for a review). As nalmefene is a non-specific antagonist and can cross the blood brain barrier, experiments with nalmefene do not suggest where or by which receptor types endogenous opioids may act to inhibit AVT release.

MR2266 slightly, but not significantly, reduced the duration of stress-induced oviposition delays. Although often considered to be a specific kappa antagonist,

MR2266 also has a high affinity for mu-receptors (Magnan *et al,*. 1982). Consequently, the interpretation of the present finding with respect to receptor types is somewhat ambiguous. One interpretation is that mu receptors mediate oviposition delays and the slight reduction in delay duration was due to some mu-receptor antagonism. This explanation is consistent with the observation that norBNI, a specific kappa receptor antagonist, did not reduce delay duration. Another interpretation is that oviposition delays are mediated by kappa receptor pathways and the slight reduction in delay duration was due to antagonism of these receptors. Possibly both kappa and mu receptors are involved in delaying oviposition. That MR2266 did not significantly reduce delay duration may be because the dose (2 mg/kg) was too low to adequately antagonise the relevant receptors (mu or kappa). The same dose of MR2266 (2 mg/kg) suppresses stereotypic pecking by broiler breeder fowls for only a short time, with no effects evident 30-45 min after injection (Kostal & Savory, 1994). Hence, MR2266 might have a relatively short half-life in hens and this might have contributed to its lack of effect on oviposition delay duration. If this is the case then MR2266 injections nearer the expected oviposition time than those in the present experiment might cause a greater reduction in delay duration.

NorBNI did not reduce the duration of stress-induced oviposition delays. As the posterior pituitary lies outside the blood brain barrier, and so is exposed to circulating norBNI, this finding suggests that peripheral kappa receptors do not have an important role in the causation of the delays. This is consistent with the suggestion that opioid receptors are not associated with the terminals of the avian AVT neurone (see Section 1.6.1). Although norBNI crosses the blood brain barrier only poorly, an i.v. dose similar to that used here completely prevents the disruption to parturition of rats caused by the selective kappa receptor agonist U50,488 (Douglas *et al.*, 1993). As U50,488 can inhibit oxytocin release both centrally and peripherally (Pumford *et al.*, 1993; Russell *et al.*, 1993), this systemic dose of norBNI is presumably able to antagonise central kappa receptors in the rat. Thus, the present study may also suggest that central kappa receptors do not have a major role in the causation of oviposition delays during stress.

If the opioidergic mechanism which mediates oviposition delays does not involve kappa-receptors, then presumably it involves mu and/or delta receptors. Mu but not delta opioid receptors have been implicated in the opioidergic control of oxytocin release in mammals (Bicknell, 1985) and so, by analogy, it may be mu rather than delta receptor pathways that delay oviposition. A specific delta receptor antagonist, such as naltrindole (McKnight & Rees, 1991), could be used to confirm that these receptors are not involved.

The mean duration of stress-induced oviposition delay following saline injection was shorter in Section 7.3 (29.0 min) than in Sections 7.2, 7.4 and 7.5 (82.3, 92.0 and 86.4 min, respectively). In contrast to Sections 7.2, 7.4 and 7.5, the hens in Section 7.3 had been housed in groups in floor pens until three weeks before they were used in the current experiment, and this may have reduced the stress associated with relocation from individual to group cages. Additionally, as ISA Brown hens were used in Sections 7.2, 7.4 and 7.5 and Hisex hens in Section 7.3, the differences in delay duration could be related to the strain of hen used. Although the different group sizes used in some of these experiments may also contribute towards the different durations of oviposition delays, this is likely to be a minor factor as the group sizes in Sections 7.3. and 7.5. were the same, yet the delays were longer in the latter.

In conclusion, the present experiments indicate that endogenous opioids are involved in the mechanisms underlying stress-induced oviposition delays in hens. Although peripheral kappa receptors are not responsible for these delays, central mu and kappa receptors may be.

# CHAPTER 8: **Effects of Exogenous Opioid Agonists on Oviposition Time**

## 8.1 Introduction

It is hypothesised that stress-induced oviposition delays are the result of an opioid mediated inhibition of AVT release from the posterior pituitary (see General Introduction). The finding that treatment with an opioid receptor antagonist, nalmefene, reduces the duration of these delays supports this hypothesis (Chapter 7). If stress does act via opioidergic pathways to induce oviposition delays then it should be possible to induce similar delays by administering exogenous opioid receptor agonists. This chapter investigated the effects of two such agonists, morphine and U50,488, on oviposition time.

Morphine, a specific mu receptor agonist (see Section 1.6.1), inhibits neurohypophysial oxytocin release in mammals, thus prolonging parturition and disrupting lactation (Haldar & Sawyer, 1978; Russell & Spears, 1983; Russell *et al.*, 1989). In mammals, morphine acts centrally to inhibit neurohypophysial hormone release (e.g. Pumford *et al.*, 1991). Morphine has often been used as an opioid agonist in studies of nociception in domestic fowls and has strain dependent analgesic and hyperalgesic effects which are naloxone reversible (Hughes, 1990). Although morphine inhibits osmotically stimulated AVT release in day-old chicks and in laying hens (Xu *et al.*, 1991; Dublecz *et al.*, 1992), its effects on oviposition have not been described previously.

In rats, the selective kappa opioid receptor agonist U50,488 (see Section 1.6.1 and Von Voigtlander *et al.*, 1983) inhibits neurohypophysial oxytocin release, prolonging parturition, and inhibits AVP release (Oiso *et al.*, 1988; Douglas *et al.*, 1993). In mammals, U50,488 acts both centrally and peripherally to inhibit neurohypophysial hormone release (Zaho *et al.*, 1988a; Pumford *et al.*, 1993; Russell *et al.*, 1993). U50,488 has been used as a specific agonist of kappa receptors of chick neurones *in vitro,* where it is antagonised by norBNI (Fletcher & Chiappinelli, 1993). Effects of U50,488 on oviposition in hens have not been described previously.

As delta opioid receptors have not been implicated in the opioidergic control of oxytocin release in mammals (Bicknell, 1985, 1993), the effects of a delta opioid receptor agonist were not investigated in this study.

## 8.2 Effects of a Range of Doses of Morphine on Oviposition Time

The aim of this experiment was to investigate the effects of morphine, at a range of doses, on hens' oviposition times. From this Experiment, a dose of 6 mg/kg morphine was chosen for further testing in Section 8.3.

### 8.2.1 Materials and Methods

From 35 stock hens aged 40 weeks, 26 whose next oviposition times could be predicted were selected over three days and allocated randomly to receive an injection of either 0.9% saline (n = 6) or 2.5, 5 or 10 mg/kg morphine (n = 5, 8 and 7). This range includes doses found to inhibit the milk ejection reflex in mice and rats (Haldar & Sawyer, 1978; Russell & Spears, 1983) and to prolong labour in rats (Russell *et al.*, 1989), and doses in excess of those found to inhibit osmotically stimulated AVT release in both chicks and laying hens (Xu *et al.*, 1991; Dublecz *et al.*, 1992). Morphine (morphine sulphate, Sigma) was dissolved in 0.9% saline (10 mg/ml) and stored at room temperature until required. This solution was injected at 0.25, 0.5 and 1 ml/kg for doses of 2.5, 5 and 10 mg/kg morphine, respectively. Saline injections were 0.5 ml/kg. All injections were i.v., 40 min before predicted oviposition time.

### 8.2.2 Results

Four hens (1 x 2.5 mg/kg, 2 x 5 mg/kg and 1 x 10 mg/kg morphine treatments) were excluded from the analysis as their egg sequences ended within two days of the injection, thus preventing accurate calculation of expected oviposition times.

The mean Δ oviposition time (see Section 2.3 for definition and calculation of this) for each treatment is shown in Table 8.1. There were no significant differences between any pairs of treatments (all $P>0.05$ by t-test). However, the SEM tends to increase as the dose rises, indicating that the Δ oviposition time is more variable following higher doses of morphine. To investigate this trend, the effect of morphine on individual ovipositions was examined.

Figure 8.1 shows the time from injection until expected oviposition time and the time from injection until observed oviposition time for each hen. As described previously (Section 2.3), hens were all injected at the same interval before predicted oviposition times and then expected oviposition times, more accurate estimates of

| Injection | Mean Δ oviposition time (min) | |
|---|---|---|
| Saline | 7.5 | (7.9) |
| 2.5 mg/kg Morphine | -2.0 | (6.6) |
| 5 mg/kg Morphine | -4.5 | (13.9) |
| 10 mg/kg Morphine | -11.7 | (15.6) |

**Table 8.1** Mean (±SEM) Δ oviposition time after injection of either saline (n = 6) or 2.5, 5 or 10 mg/kg morphine (n = 4, 6 & 6).

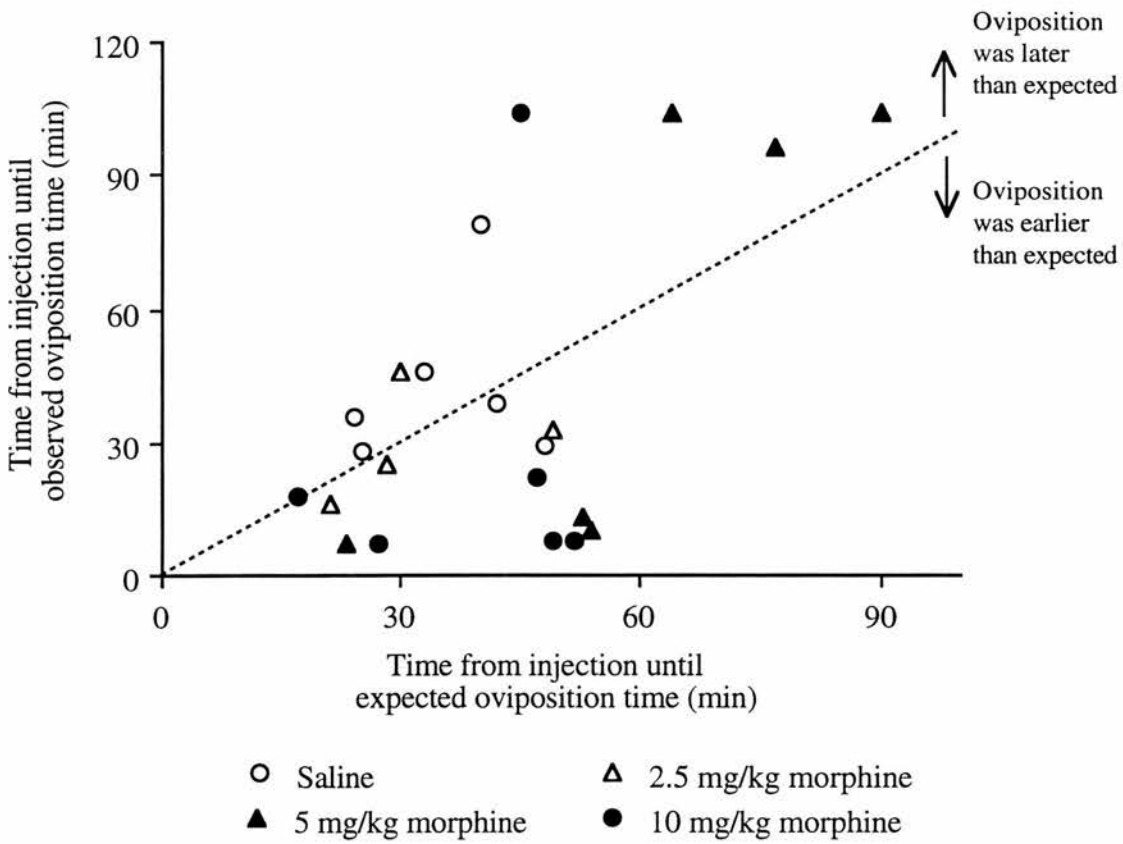



**Figure 8.1** The time from injection, of either saline or 2.5, 5 or 10 mg/kg morphine, until expected oviposition time and the time from injection until observed oviposition time for each hen. The diagonal dashed line indicates the expected oviposition time relative to the two axes. Points below this line indicate ovipositions that occurred earlier than expected and points above this line indicate ovipositions that occurred later than expected.

when oviposition would normally have occurred, were calculated a day later. As expected oviposition times usually differed slightly from predicted oviposition times, there was a range of times from injection until expected oviposition time in this experiment. From Figure 8.1, it can be seen that 3 of the 6 hens receiving 5 mg/kg morphine and 3 of the 6 hens receiving 10 mg/kg morphine laid earlier than expected and within 15 min of these injections. As the sample sizes in this experiment were small and the injections were relatively close to expected oviposition time, it is not clear if 5 and 10 mg/kg morphine injections can act to induce some ovipositions prematurely or if the distribution of oviposition times simply reflects random variation. Similarly, it is not clear if 5 or 10 mg/kg morphine injections delay oviposition when they do not induce it prematurely. Injections of saline or 2.5 mg/kg morphine did not appear to affect oviposition time.

## 8.3 Effects of 6 mg/kg Morphine on Oviposition Time

In Section 8.2, injections of 5 or 10 mg/kg morphine appeared to induce some ovipositions prematurely. However, as the sample sizes were small and the injections were administered relatively close to expected oviposition time, it is not clear whether the apparent oviposition inducing effect of morphine was real or due to random variation. The aim of the following experiment was to investigate the effects of 6 mg/kg morphine on oviposition time. Larger sample sizes than those in Section 8.2 were used and, in order that any ovipositions inductions would be more obvious, injections were administered earlier relative to predicted oviposition time.

### 8.3.1 Materials and Methods

From 34 stock hens aged 44 weeks, 23 whose next oviposition times could be predicted were selected over six days and allocated randomly to receive an injection of either 0.9% saline (n = 9) or 6 mg/kg morphine (n = 14). It was suspected from Section 8.2 that morphine may have two effects on oviposition time, i.e. it either induces oviposition prematurely or does not. To increase the chance of detecting such a bimodality, more hens were allocated to the morphine treatment than to the saline treatment. Morphine was dissolved in 0.9% saline (12 mg/ml) and stored at room temperature until required. Morphine and saline were both injected i.v. at 0.5 ml/kg, 75 min before predicted oviposition time. The dose of 6 mg/kg morphine was within the range of doses which had the greatest influence on oviposition time in Section 8.2.

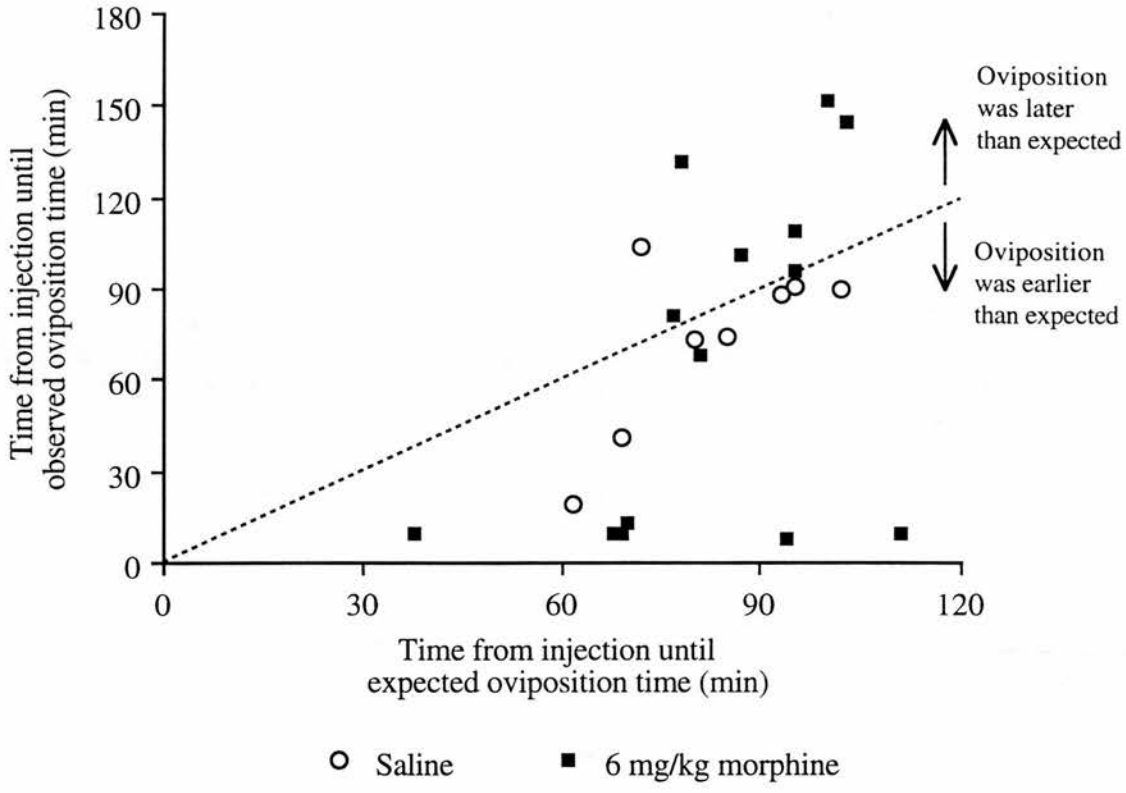



**Figure 8.2** The time from injection, of either saline or 6 mg/kg morphine, until expected oviposition time and the time from injection until observed oviposition time for each hen. The diagonal dashed line indicates expected oviposition time relative to the two axes. Points below this line indicate ovipositions that occurred earlier than expected oviposition time and points above this line indicate ovipositions that occurred later than expected oviposition time.

### 8.3.2 Results

One hen (saline treatment) was excluded from the analysis as its egg sequence ended within two days of injection, thus preventing accurate calculation of expected oviposition time.

Figure 8.2 shows the time from injection until expected oviposition time and the time from injection until observed oviposition time for each hen. As described in Section 8.2.2, there was a range of times from injection until expected oviposition

time. Of the 14 hens receiving morphine, 6 laid 8-13 min (mean = 10 min) after injection, and these ovipositions were 28-101 min (mean = 65 min) before their expected times. As these ovipositions were premature and occurred at a relatively short and regular interval after injection, it appears that morphine may have induced them. The remaining 8 hens receiving morphine laid either around the expected time (n = 5) or somewhat later (n = 3). No ovipositions occurred within 15 min of a saline injection.

If morphine did induce oviposition prematurely in some hens but not in others, then the distribution of observed oviposition times, relative to expected oviposition times and injection times, is not expected to be unimodal. Therefore, the aim of the following analysis was to test the data for unimodality. If morphine induced some ovipositions prematurely then the observed times of these should be related to injection times. Conversely, observed times of other ovipositions (non-induced) might be related to their expected times. To allow for the variation in injection time, a score was calculated for each oviposition by dividing the time from injection until observed oviposition time by the time from injection until expected oviposition time. Thus, premature ovipositions have a score less than one, ovipositions occurring at the expected time have a score equal to one and delayed ovipositions have a score greater than one. These scores for both morphine and saline injected hens are shown in Figure 8.3.

To test the null hypothesis that the distribution of the scores of the morphine treated hens was unimodal, the data were analysed using the procedure of Silverman (1981; see also Sinnett-Smith & Waddington, 1992). Briefly, the distribution of the 14 scores was smoothed by overlaying each score with a normal distribution of fixed variance. Changing the variance alters the amount of smoothing and increasing the amount of smoothing results in a distribution with fewer modes. The distribution of the 14 scores was smoothed just enough to generate a unimodal distribution (i.e. any less smoothing would produce a bimodal distribution). From this generated distribution, 500 simulated random samples of 14 values were drawn, and each of these was smoothed using the same variance that gave the distribution from the original scores.

As only 8 of the 500 simulations (1.6%) had a unimodal distribution when smoothed, the probability that the original scores were distributed unimodally is 0.016. As 348 of the 500 simulations (69.6%) were distributed bimodally, the probability that the original scores were distributed bimodally is 0.696. Hence, the probability that the distribution has more than two modes is 0.288 (1-(0.696+0.016) = 0.288). Thus, it was concluded that the distribution of oviposition times following

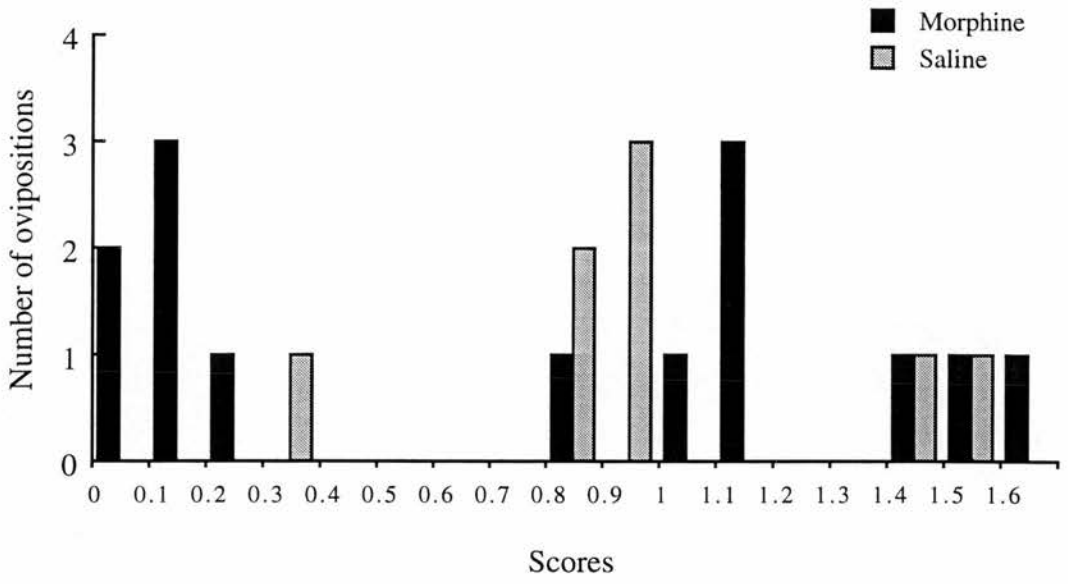



**Figure 8.3** Distribution of scores for hens receiving an injection of either 6 mg/kg morphine or saline. For each hen the score was calculated by dividing the time from injection until observed oviposition time by the time from injection until expected oviposition time (see text, p.93). The six hens whose ovipositions were induced by morphine all have scores less than 0.3.

morphine injection was not unimodal ($P<0.05$) but may be bimodal ($P>0.05$). The finding that the distribution was not unimodal supports the hypothesis that morphine has more than one effect on oviposition time (i.e. it does or does not induce oviposition prematurely).

For the saline treated hens, there were too few scores (n = 8) to permit an analysis of their distribution using the Silvermann (1981) procedure. However, for these hens there is no suggestion of bimodallity in the distributions of either oviposition delay durations (Figure 8.2) or scores (Figure 8.3).

Although systematic behavioural observations were not made, it was noted that hens whose ovipositions were induced prematurely by morphine injection tended to show characteristic pre-laying behaviours, such as pacing and sitting, at expected oviposition time.

For the following analysis the six ovipositions which occurred prematurely and within 15 min of the morphine injection are considered to have been induced by this treatment (see above analysis). There was no significant difference in the times from

morphine injection until expected oviposition time between the ovipositions which were induced and those which were not (t-test; t=1.34, 6df, $P>0.05$).

The mean Δ oviposition times for hens receiving saline and for those receiving morphine but whose ovipositions were not induced by this treatment are shown in Table 8.2. The mean Δ oviposition time following injection of morphine was significantly longer than that following injection of saline (t-test; t=2.73, 13df, $P<0.05$). If it is assumed that the hens whose ovipositions were induced by morphine were a random sample of the population (see Discussion), this result suggests that morphine delays oviposition in some birds when it does not induce it.

| Injection | Mean Δ oviposition time (min) | |
|---|---|---|
| Saline | -9.8 | (7.6) |
| Morphine | 21.9 | (8.7) |

**Table 8.2** Mean (±SEM) Δ oviposition time after saline injection (n = 8) or 6 mg/kg morphine injection where this did not induce oviposition (n = 8).

## 8.4 Effects of a Range of Doses of U50,488 on Oviposition Time

The aim of this experiment was to investigate the effects of U50,488, at a range of doses, on hens' oviposition times. From this experiment a dose of 4 mg/kg U50,488 was chosen for further testing in Section 8.5.

### 8.4.1 Materials and Methods

From 35 stock hens aged 40 weeks, 24 whose next oviposition times could be predicted were selected over two days and randomly allocated to receive an injection of either 0.9% saline (n = 5) or U50,488 at 1, 2, 4 or 5 mg/kg (n = 5, 8, 4 and 2). This range of U50,488 doses includes some that, when administered systematically to rats, inhibit electrical activity of putative oxytocin neurones, inhibit AVP release and prolong labour (Clarke & Wright, 1989; Douglas *et al.*, 1993; Pumford *et al.*, 1993). U50,488 (*trans*-(+)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulphonate salt; Research Biochemicals Incorporated, Natick, MA) was dissolved in 0.9% saline (4 mg/ml) and stored at 4°C until required.

This solution was injected at 0.25, 0.5, 1.0 and 1.25 ml/kg for doses of 1, 2, 4 and 5 mg/kg U50,488, respectively. Saline injections were 0.5 ml/kg. All injections were i.v., 40 min before predicted oviposition time. Mean Δ oviposition times were compared between treatments by t-test.

### 8.4.2 Results

Two hens (1 x 2 mg/kg and 1 x 4 mg/kg U50,488 treatments) were excluded from the analysis as their egg sequences ended within two days of injection, thus preventing accurate calculation of expected oviposition times.

Mean Δ oviposition time for each of the treatments and the statistical comparasons between these are shown in Figure 8.4. For each U50,488 dose, mean Δ oviposition time was positive, indicating that treatment with U50,488 tends to cause oviposition delays. Injection of 4 mg/kg U50,488 caused the greatest delay relative to saline treatment (Figure 8.4).

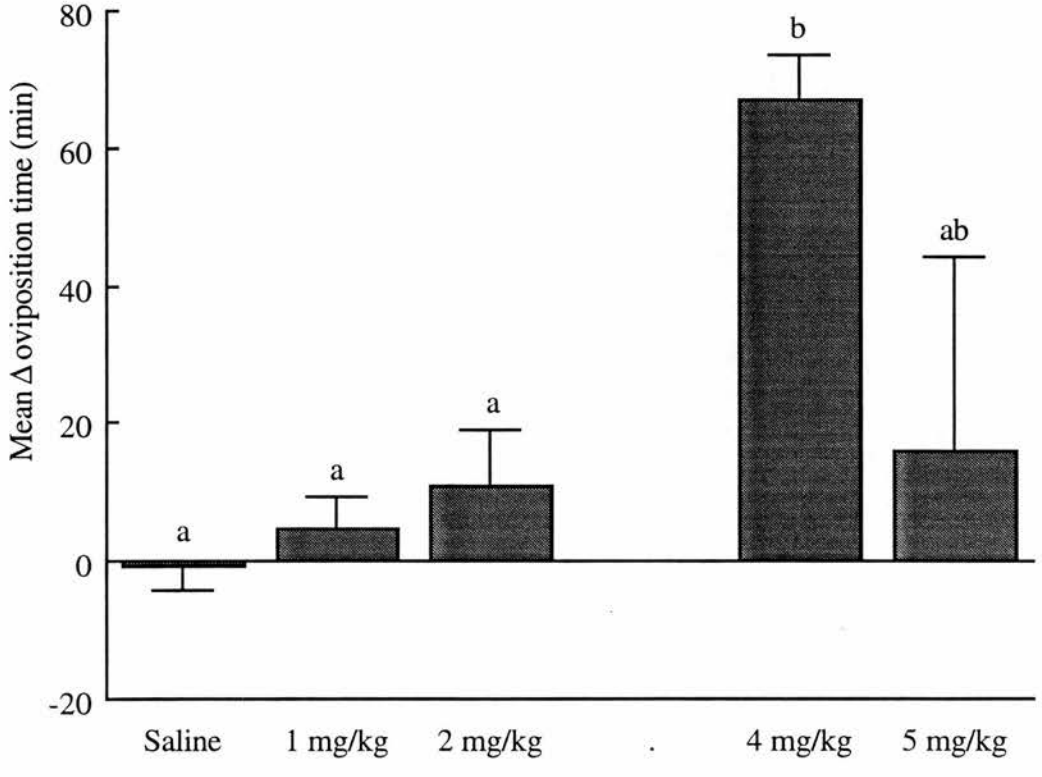



**Figure 8.4** Mean (±SEM) Δ oviposition times following injection of either saline (n = 5) or U50,488 at 1, 2, 4 or 5 mg/kg (n = 5, 7, 3 and 2). Means with no common letter differ significantly ($P<0.01$) and means with a common letter do not differ significantly ($P>0.05$).

## 8.5 Effects of 4 mg/kg U50,488 on Oviposition Time

In Section 8.4, treatment with U50,488 delayed oviposition time, 4 mg/kg causing the longest delays. The aim of the following experiment was to confirm, through the use of a larger sample size, that 4 mg/kg U50,488 injections delay oviposition.

### 8.5.1 Materials and Methods

From 35 stock hens aged 47 weeks, 23 whose next oviposition times could be predicted were selected over two days and randomly allocated to receive an injection of either 0.9% saline (n = 12) or 4 mg/kg U50,488 (n = 11). U50,488 was dissolved in 0.9% saline (5 mg/ml) and stored at 4°C until required. U50,488 and saline were both injected i.v. at 0.8 ml/kg, 30 min before predicted oviposition time.

### 8.5.2 Results

One hen (saline treatment) laid as it was restrained for injection, another (U50,488 treatment) laid before it was due to be injected and a third (U50,488 treatment) did not receive its full injection volume. These three hens were excluded from the analysis.

Mean Δ oviposition times after injections of either saline or 4 mg/kg U50,488 are shown in Table 8.3. The mean Δ oviposition time after 4 mg/kg U50,488 injection was significantly longer than that after saline injection (t-test; t=2.76, 4 df, $P<0.05$). This confirms that injection of 4 mg/kg U50,488 delays oviposition.

| Injection | Mean Δ oviposition time (min) | |
|---|---|---|
| Saline | 5.0 | (6.4) |
| 4 mg/kg U50,488 | 26.4 | (4.4) |

**Table 8.3** Mean (±SEM) Δ oviposition time after injection of either saline (n = 11) or 4 mg/kg U50,488 (n = 9).

## 8.6 Does Treatment with Nalmefene Reduce the Duration of Oviposition Delays Induced by U50,488?

In Sections 8.4 and 8.5, injections of 4 mg/kg U50,488 caused oviposition delays. U50,488 is known to prolong labour in rats partly by directly inhibiting uterine contractions in a non opioidergic manner and partly by inhibiting neurohypophysial oxytocin release in an opioidergic manner (Douglas *et al.*, 1993). It is not known if U50,488 delays oviposition by direct uterine effects or through its actions as an opioid receptor agonist. If U50,488 delays oviposition by acting as an opioid agonist then these delays should be prevented by treatment with an opioid antagonist. The aim of the following experiment was to see if co-treatment with the non-specific opioid antagonist nalmefene would reduce the duration of oviposition delays induced by U50,488 injection.

### 8.6.1 Materials and Methods

From 48 stock hens aged 27 weeks, 30 whose next oviposition times could be predicted were selected over 5 days and randomly allocated to receive one of four injections. These were 0.9% saline, 4 mg/kg U50,488, 4 mg/kg U50,488 + 0.5 mg/kg nalmefene, and 0.5 mg/kg nalmefene (n = 8, 7, 7 and 8, respectively). This dose of nalmefene reduces the duration of stress-induced oviposition delays (Chapter 7). U50,488 was dissolved in 0.9% saline (8 mg/ml and 16 mg/ml) and stored at 4$^{o}$C until required. Nalmefene was dissolved in 0.9% saline (1 mg/ml and 2 mg/ml) and stored at -18$^{o}$C until required. Shortly before injection of U50,488 + nalmefene, equal volumes of 16 mg/ml U50,488 and 2 mg/ml nalmefene were mixed to give concentrations of 8 mg/ml and 1 mg/ml, respectively. All injections were 0.5 ml/kg i.v., 30 min before predicted oviposition time. Mean durations of oviposition delay were compared between treatments by t-test (pooled where standard deviations were similar).

### 8.6.2 Results

Mean durations of oviposition delay following each of the four injection treatments and the statistical comparisons between these are shown in Figure 8.5. Oviposition delays following injection of U50,488 were significantly longer than those following any of the other three injection treatments. Thus, 4 mg/kg U50,488 induced oviposition delays but this response was reduced by co-treatment with nalmefene.

There were no significant differences between saline, U50,488 + nalmefene and nalmefene treatments. Thus, compared with the saline controls, 4 mg/kg U50,488 in the presence of 0.5 mg/kg nalmefene did not induce oviposition delays. Furthermore, treatment with nalmefene alone did not alter oviposition time relative to saline treatment.

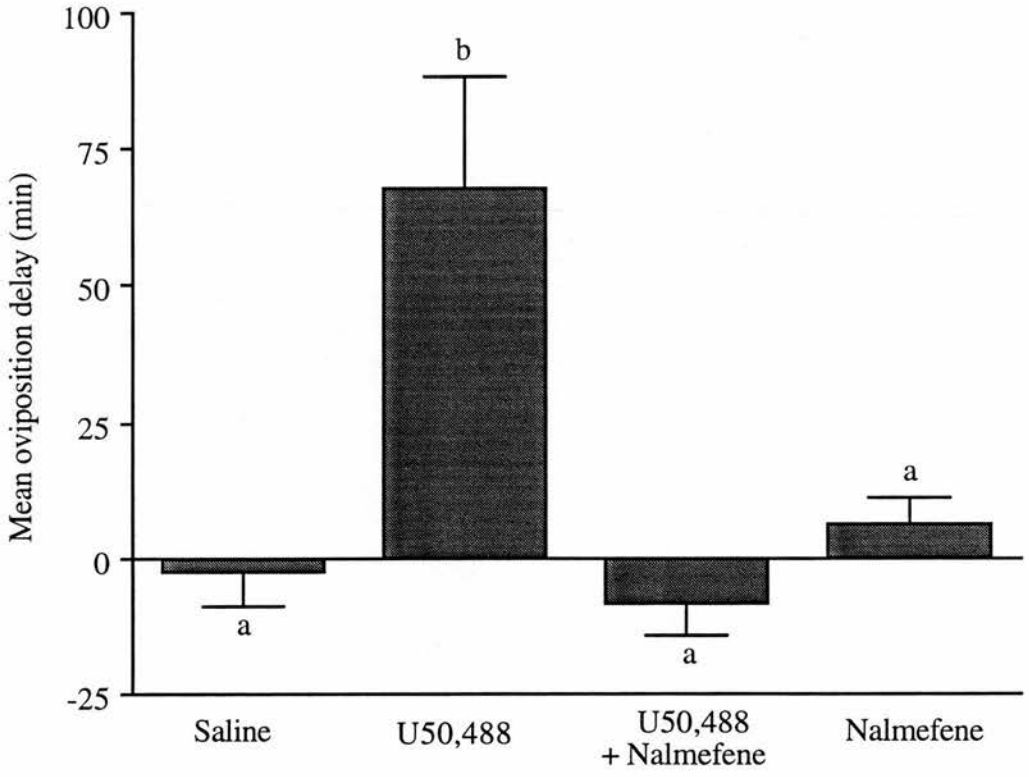



**Figure 8.5** Mean (±SEM) durations of oviposition delay following injection of either saline (n = 8), 4 mg/kg U50,488 (n = 7), 4 mg/kg U50,488 + 0.5 mg/kg nalmefene (n = 7) or 0.5 mg/kg nalmefene (n = 8). Means with no common letter differ significantly ($P<0.05$).

## 8.7 Do AVT Injections End the Oviposition Delaying Effect of U50,488?

Injections of 4 mg/kg U50,488 cause oviposition delays in a nalmefene reversible manner (Sections 8.4, 8.5 and 8.6). In mammals, U50,488 disrupts parturition and it is hypothesised that this is due to inhibition of oxytocin release (Douglas *et al.*, 1993). This hypothesis is supported by the finding that these disruptions are associated with reduced plasma oxytocin concentrations and are ended by oxytocin injection (Douglas *et al.*, 1993). Possibly U50,488 delays oviposition by inhibiting neurohypophysial AVT release. If this hypothesis is correct, then injecting exogenous AVT should end these delays and allow oviposition to proceed. The aim of the following experiment was to see if the oviposition delaying effect of U50,488 can be ended by injecting AVT.

### 8.7.1 Materials and Methods

From 25 Hisex hens aged 31 weeks and reared as described in Section 7.3, 20 whose next oviposition times could be predicted were selected and randomly allocated to receive an injection of either 0.9% saline or 0.1 ug/kg AVT (n = 10 each), each following an injection of 4 mg/kg U50,488. This dose of AVT ends stress-induced oviposition delays (Chapter 6). U50,488 was dissolved in 0.9% saline (8 mg/ml) and stored at 4$^{o}$C until required. U50,488 solution was injected i.v. (0.5 ml/kg) 30 min before predicted oviposition time. Twenty min after predicted oviposition time each hen received a second injection: either saline or AVT (as described in Section 6.3) and the time from this until observed oviposition, the oviposition induction time, was recorded. From the experiments described in Sections 8.4, 8.5 and 8.6 it was expected that most hens receiving 4 mg/kg U50,488 would still be carrying their egg 20 min after predicted oviposition time.

### 8.7.2 Results

The egg sequences of three hens (2 x saline treatment, 1 x AVT treatment) ended within one day of treatment, thus preventing accurate calculation of their expected oviposition times These hens and another (saline treatment) which did not carry an egg on the day of treatment were excluded from the analysis.

Three of the seven remaining hens allocated to receive saline and six of the nine remaining hens which had been allocated to receive AVT laid before they were due to

receive these injections (i.e. before 20 min after predicted oviposition time). Thus only four hens received a saline injection and three hens an AVT injection. Mean times from saline and AVT injections until observed ovipositions are shown in Table 8.4. The time from injection until observed oviposition was significantly shorter for AVT injection than for saline injection (t-test; t=3.86, 3df, $P<0.05$). The mean oviposition delay for all seven birds allocated to receive saline was 17.4 min (± 8.6 SEM).

| Injection | Mean oviposition induction time (min) |
|---|---|
| Saline | 23.1 (5.3) |
| AVT | 2.5 (0.3) |

**Table 8.4** Mean (±SEM) oviposition induction times following injection of either saline (n = 4) or AVT (n = 3) 20 min after the predicted oviposition times of hens treated with 4 mg/kg U50,488.

## 8.8 Discussion

As some birds receiving 6 mg/kg morphine i.v. laid prematurely and shortly after this injection, it appears that morphine can act to induce some ovipositions prematurely. The distribution of observed oviposition times following morphine injection was not unimodal, but may be bimodal, supporting the conclusion that this treatment has more than one effect, i.e. it does or does not induce oviposition. Hens whose ovipositions were considered to have been induced prematurely by injection of morphine performed pre-laying behaviours at expected oviposition time, confirming that these ovipositions were indeed premature. Saline injections did not appear to induce premature oviposition in any of the experiments described in this chapter.

The finding that morphine induces some ovipositions prematurely was unexpected. In rats, similar i.v. doses of morphine inhibit release of neurohypophysial oxytocin and so prolong parturition (Russell *et al.*, 1989; Pumford *et al.*, 1991; Pumford *et al.*, 1992). Morphine also disrupts the milk-ejection reflex in both mice and rats, a process known to be dependent on oxytocin release (Haldar & Sawyer, 1978; Russell & Spears, 1983). Furthermore, at doses lower than those tested here, i.v. morphine inhibits osmotically stimulated AVT release in chicks and in

laying hens (Xu *et al.*, 1991; Dublecz *et al.*, 1992). Consequently, it had been predicted that morphine would induce oviposition delays by inhibiting neurohypophysial AVT release.

Although some ovipositions which were not induced by morphine were delayed relative to those of saline treated hens, this finding must be interpreted cautiously. As it is not known why morphine induced some ovipositions, it is not known if these represented a random sample of the treated hens. Therefore, the duration of oviposition delays of non-induced ovipositions may not be representative of all hens. For example, morphine may have induced oviposition in hens which would have laid slightly earlier than expected on the day of treatment (had there been no treatment), but not in hens which would have laid slightly later than expected. If this happened then the mean Δ oviposition time of the morphine treated hens whose ovipositions were not induced would be greater than that for the control hens, even though morphine did not actually delay oviposition. If it is assumed that the hens in which oviposition was induced by morphine were a random sample of the population, the results would indicate that morphine acts to delay some ovipositions when it does not induce them. As described above, morphine inhibits oxytocin release in mammals and inhibits osmotically stimulated AVT release in chicks and hens. Therefore, morphine might delay oviposition by inhibiting neurohypophysial AVT release.

It is not known by what mechanism morphine acts to induce some ovipositions prematurely, or even if it is acting as an opioid agonist. As the hens whose ovipositions were induced and those whose ovipositions were not induced were injected at similar times (relative to expected oviposition time), the mechanism by which morphine acts to induce oviposition does not appear to be related to the time of injection. It is also not known if particular hens are more succeptable than others to this effect of morphine, or if it occurs with equal probability in all birds.

Morphine is reported to have more than one effect on the release of substance P from rat trigeminal nucleus slices. Morphine inhibits release at low and medium concentrations but facilitates release at intermediate (i.e. between low and medium) and higher concentrations (Suarez-Roca *et al.*, 1992). As various specific receptor antagonists inhibit different parts of morphine's multiphasic effect on substance P release, it is thought that morphine may act via different receptor types to cause these different effects, and that the effects are dose related because morphine has different affinities for each receptor type (Suarez-Roca & Maixner, 1992). Perhaps, by acting on more than one opioid receptor type, morphine has both facilitatory and inhibitory effects on AVT release and so can induce oviposition (by facilitating release) or delay oviposition (by inhibiting release). This hypothesis would be supported if it was

shown that the separate oviposition inducing and delaying effects of morphine were differentially prevented by specific antagonists of different opioid receptor types. Although U50,488 generally inhibits electrical activity of oxytocin neurones *in vivo*, it sometimes increases activity at low concentrations, opposite effects which may be associated with its actions on different sub-types of the kappa opioid receptor (Pumford *et al.*, 1993). As different subtypes of a receptor may be linked to different G-proteins, and so may have different effects when stimulated, an opioid agonist can affect neuronal activity in opposite ways (Shen & Crain, 1989). Perhaps the oviposition inducing and delaying effects of morphine are mediated by different subtypes of one opioid receptor type. It has been reported that morphine occasionally has a stimulatory effect on the activity of putative oxytocin neurones in the rat supraoptic nucleus (Leng *et al.*, 1990; Pumford *et al.*, 1991).

An alternative possibility is that morphine acts directly on uterine muscle to promote contractions which expel some eggs prematurely. Widy-Tyszkiewicz *et al.* (1978) found that morphine caused small dose dependent increases in contractions of isolated rat uteri, while Acevedo and Contreras (1987) observed that morphine enhanced, in a non-opioid manner, the contraction inducing effects of adrenaline on uteri that had been isolated from mice which had been chronically exposed to morphine. By contrast, morphine has also been reported to have no effect on either spontaneous or oxytocin induced contractions of isolated rat uteri (Russell *et al.*, 1989), and to slightly inhibit the stimulated contractions of *in vitro* uteri from non-pregnant rats and mice (Acevedo & Contreras, 1984; Sivalingam & Pleuvry, 1985). If opioid receptor antagonism does not prevent the premature induction of oviposition by morphine, then this effect may be due to non-opioid actions of morphine on the uterus. This possibility could be tested by investigating the effects of morphine on hens' uterine contractions, *in vivo* and *in vitro*.

The main conclusion from the experiments involving U50,488 is that this kappa receptor agonist can induce oviposition delays in undisturbed hens. This effect was prevented by nalmefene, thus confirming that U50,488 acted as an opioid agonist. The finding that such an exogenous agonist induces oviposition delays supports the hypothesis that stress-induced oviposition delays could be mediated by endogenous opioids.

In anaesthetised rats, U50,488 reduces the firing rate of putative oxytocin neurones in the supraoptic nuclei and inhibits electrically stimulated oxytocin release (Pumford *et al.*, 1993; Russell *et al.*, 1993). Thus, U50,488 can inhibit oxytocin release by acting in the hypothalamus (centrally) and in the neurohypophysis (peripherally). As kappa receptors have not been associated with the terminals of AVT

neurones (see Section 1.6.2) it is unlikely that U50,488 inhibits AVT release at the level of the posterior pituitary. Therefore, U50,488 may act centrally to inhibit neurohypophysial AVT release and hence delay oviposition. Although the route through which uterine contractions stimulate AVT release has not been described, it may involve a spinal reflex similar to that which triggers oxytocin release in mammals during parturition and lactation (Edquist & Stabenfeldt, 1993). Therefore, a further possibility is that U50,488 acts within the spinal cord to suppress the passage of excitatory stimuli from the uterus to the hypothalamus, and so to inhibit AVT release. Morphine can act on spinal sites to inhibit the milk ejection reflex in lactating rats, a process dependent on oxytocin release (Wright, 1985). As U50,488 can cross the blood brain barrier, the experiments reported here do not indicate where U50,488 may be acting to inhibit AVT release.

It should be noted that the dose of U50,488 required to delay oviposition (4 mg/kg) was higher than those doses required to inhibit release of oxytocin and AVP and to disrupt parturition in mammals (Oiso *et al.*, 1988; Douglas *et al.*, 1993; Pumford *et al.*, 1993; Russell *et al.*, 1993). As the dose used was relatively high, the possibility that U50,488 may have had non-specific activity which contributed towards the effects reported here cannot be excluded.

The finding that AVT injection ends the oviposition delaying effect of U50,488 (Section 8.7) is consistent with the hypothesis that these delays are caused by inhibition of AVT release. The hypothesis that U50,488 inhibits AVT release is tested in Chapter 9.

A further finding in this chapter is that nalmefene injection alone does not alter the oviposition time of undisturbed hens compared with saline injection (Section 8.6). This suggests that inhibitory opioidergic pathways do not control oviposition time in undisturbed hens.

In Section 8.7, U50,488 did not appear to induce oviposition delays as potently as in the preceding experiments (Sections 8.4, 8.5 and 8.6). Perhaps the Hisex strain of hen in Section 8.7 and the ISA Brown strain in Sections 8.4, 8.5 and 8.6 respond differently to U50,488, and this was responsible for the differences in delay duration. Differences in duration of oviposition delay induced by social stress were also found between these strains (Chapter 7).

To summarise, U50,488 had a delaying effect on oviposition, and this was prevented by nalmefene and ended by exogenous AVT. Morphine induced some ovipositions prematurely but may have delayed others. These findings support the hypothesis that stress-induced oviposition delays are caused by endogenous opioids acting to inhibit AVT release.

# CHAPTER 9: **Effects of U50,488 and Nalmefene on PGF2α-Induced Premature Oviposition and Associated Changes in Plasma AVT Concentration**

## 9.1 Introduction

In mammals, endogenous opioids released in response to stress inhibit the release of neurohypophysial oxytocin and so disrupt parturition and lactation (Haldar & Bade, 1981; Petherick *et al.*, 1993). Exogenous opioid agonists, such as the kappa receptor selective U50,488, also inhibit oxytocin release, by acting in both the hypothalamus and the neurohypophysis (Zaho *et al.*, 1988a; Pumford *et al.*, 1993; Russell *et al.*, 1993), and so disrupt parturition and lactation (Clarke & Wright, 1989; Douglas *et al.*, 1993). In hens, the durations of stress-induced oviposition delays are reduced by nalmefene treatment, thus implicating endogenous opioids in the underlying mechanism (Chapter 7). Oviposition delays can also be induced by i.v. administration of U50,488 (Chapter 8), and it is hypothesised that opioidergic inhibition of neurohypophysial AVT release is involved in the mechanism underlying these delays and those induced by environmental stress. The aim of the following study was to test the hypothesis that U50,488 would inhibit, in an opioidergic manner, the release of AVT associated with PGF2α induced uterine contractions. Treatments with nalmefene, a non-selective opioid receptor antagonist, were included so that any effects of U50,488 could be attributed to its actions on opioid receptors.

PG release from the ovary is the initial event of spontaneous oviposition and triggers, by inducing uterine contractions, the release of AVT from the posterior pituitary (Shimada & Saito, 1989). Intra-uterine injection of exogenous PGF2α induces oviposition prematurely within a few minutes and this is associated with a rise in plasma AVT concentration (Saito *et al.*, 1987; Shimada *et al.*, 1987a; Murakami *et al.*, 1990). Premature ovipositions can also be induced by i.v. PGF2α (1 μg/hen), these occurring approximately 8 min after injections given 1-4 h before expected oviposition time (Goto *et al.*, 1985). In the following experiments, PGF2α injections were used to induce uterine contractions and so stimulate AVT release, and the effects of treatments with U50,488 and nalmefene on both the induction of oviposition and the rise in plasma AVT concentration were investigated.

There were two possible times at which blood samples could be taken to investigate the effect of the experimental treatments on PGF2α induced AVT release:

either a fixed time after PGF2α injection or at the time of induced oviposition. As it was expected that U50,488 would inhibit AVT release, and as AVT release following PGF2α injection contributes towards the expulsion of the egg (Murakami *et al.*, 1990), it was not known if treatment with U50,488 would prevent PGF2α injections from inducing premature oviposition. Therefore, blood samples were taken at a fixed time after the PGF2α injections in Experiment 1. Because of problems with the assay procedure, no measures of AVT concentrations in the blood samples taken during Experiment 1 were obtained, and consequently only the effects of the treatments on oviposition induction by PGF2α injection are reported. As all PGF2α injections induced ovipositions following each treatment in Experiment 1, blood samples were taken immediately after the induced ovipositions in Experiment 2 rather than at a fixed time after injection.

## 9.2 Experiment 1: Rate of Premature Oviposition Induction by Exogenous PGF2α

The aim of this experiment was to investigate the effects of treatments with U50,488 and nalmefene on both the rate of premature oviposition induction caused by PGF2α injection and the plasma AVT concentrations 5 min after these PGF2α injections. However, because of problems with the assay, no measurements of AVT concentration were obtained and only the effects of treatments on the premature induction of oviposition by PGF2α are reported.

### 9.2.1 Materials and Methods

On each of eight days, four hens whose next oviposition times could be predicted were selected from a stock of 34, between 52 and 55 weeks of age. Each day the four hens were randomly and evenly divided between four injection treatments: saline, 4 mg/kg U50,4880, 4 mg/kg U50,488 + 0.5 mg/kg nalmefene, and 0.5 mg/kg nalmefene (thus, n = 8 for each treatment). This dose of U50,488 causes oviposition delays (Chapter 8) and this dose of nalmefene reduces the duration of oviposition delays induced by both 4 mg/kg U50,488 (Chapter 8) and social stress (Chapter 7). A first blood sample to assess basal plasma AVT concentrations was taken from each hen 1.5 h before predicted oviposition time, and then the treatment injection (see section 8.6.1 for details) was administered. PGF2α (0.5 μg/kg), a dose known to induce premature oviposition within a few minutes, was then injected (see

section 6.2.1 for details) 15-26 min (mean = 20 min) later. For each bird the times from PGF2α injection until oviposition, the oviposition induction times, were recorded and a second blood sample was taken 5.0 min after this injection.

Because of problems with the assay, AVT concentrations in the blood samples taken during this experiment were not determined and only the effects of the treatments on oviposition induction times are reported.

### 9.2.2 Results

The expected oviposition times of six hens (2 x saline, 2 x U50,488, 2 x nalmefene) could not be calculated as their egg sequences ended within three days of treatment. Another hen, due to receive U50,488 + nalmefene, did not carry an egg on the day of injection. These hens and another (saline) which laid before receiving its PGF2α injection were excluded from the following analysis (thus: saline, n = 5; U50,488, n = 6; U50,488 + nalmefene, n = 7; nalmefene, n = 6).

PGF2α injections were 0.8-2.2 h (mean = 1.3 h) before expected oviposition times and induced oviposition in every hen. Mean oviposition induction times for each treatment are shown in Figure 9.1. The effects of treatments with saline, U50,488 + nalmefene and nalmefene were compared by t-test. Although the oviposition induction times tended to be longer after both U50,488 + nalmefene and nalmefene treatments than after saline treatment (Figure 9.1), only the difference between U50,488 + nalmefene and saline treatments was significant at $P<0.05$ ($t = 2.64$). As two U50,488 treated birds laid after the blood sampling procedure, which was 5.0 min after PGF2α injection, this may have influenced the timing of these ovipositions, rank statistics were used to compare the oviposition induction times after U50,488 treatment with those following other treatments. Although the oviposition induction times tended to be longer after U50,488 treatment compared with saline, there were no significant differences between the U50,488 treatment and any of the other three treatments (all $P>0.05$ by Mann-Whitney).

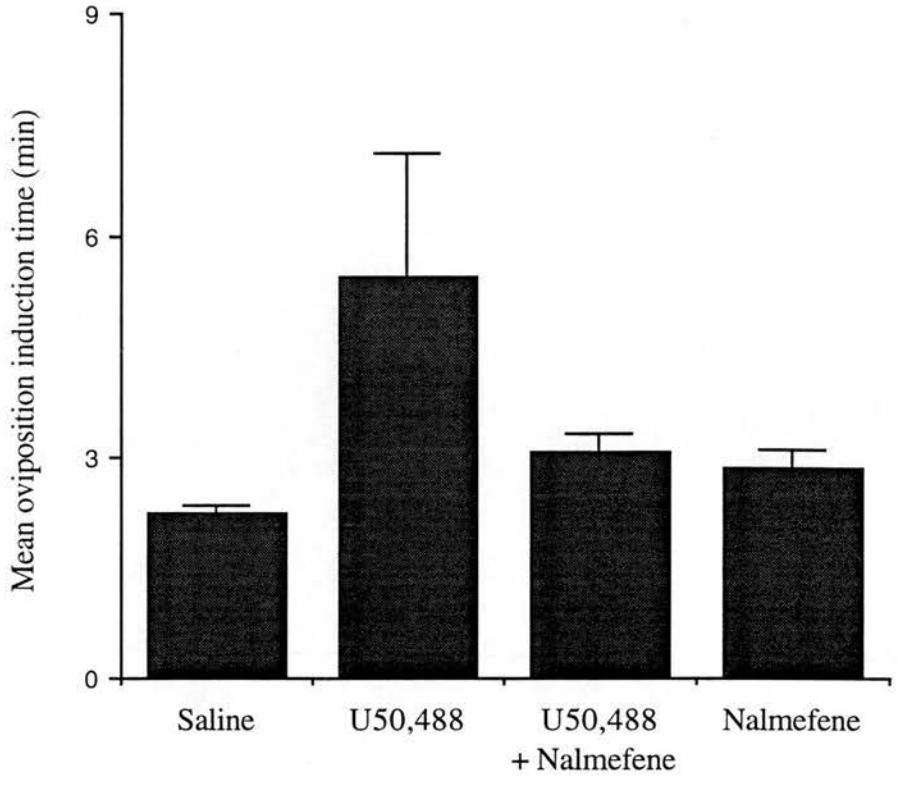



**Figure 9.1** Mean (±SEM) oviposition induction times after injection of 0.5 μg/kg PGF2α in hens treated with either saline (n = 5), 4 mg/kg U50,488 (n = 6), 4 mg/kg U50,488 + 0.5 mg/kg nalmefene (n = 7) or 0.5 mg/kg nalmefene (n = 6) in Experiment 1. Note that two ovipositions (U50,488 treatment) occurred after the blood sampling procedure 5.0 min after PGF2α injection.

## 9.3 Experiment 2: Rate of Premature Oviposition Induction by Exogenous PGF2α and Associated changes in Plasma AVT concentration

The aim of this experiment was to investigate the effects of treatments with U50,488 and nalmefene on both the rate of premature oviposition induction caused by PGF2α injection and the plasma AVT concentrations after these PGF2α injections.

### 9.3.1 Materials and Methods

Over four days a total of 24 hens (4 on each of two days and 8 on each of two days) whose next oviposition times could be predicted were selected from a stock of 32, between 27 and 32 weeks of age. On each day the hens were randomly and evenly divided between four injection treatments: saline, 4 mg/kg U50,4880, 4 mg/kg U50,488 + 0.5 mg/kg nalmefene, and 0.5 mg/kg nalmefene (thus n = 6 for each treatment). The treatments were the same as those used in Experiment 1 and are described in Section 8.6.1. These injections were given 1.5 h before predicted oviposition time and then, to assess their effects on basal plasma AVT concentrations, a first blood sample was taken 7-28 min (mean = 13 min) later. Following this blood sample each hen received i.v. 0.5 μg/kg PGF2α (for injection details see Section 6.2.1). Oviposition induction times were recorded and a second blood sample was taken from each hen immediately after induced oviposition.

AVT concentration in plasma from each blood sample was determined in duplicate using a radioimmunoassay (Peninsula Laboratories Inc.) which is described and validated in Appendix 2. Mean plasma AVT concentrations were compared between treatments by t-test (pooled where standard deviations were similar) and between blood sampling times within each treatment by paired t-test.

### 9.3.2 Results

PGF2α injections were 0.5-1.9 h (mean = 1.1 h) before expected oviposition times and induced oviposition within 25 min in all but one bird (U50,488 treatment). The mean oviposition induction times after treatments with saline, U50,488 + nalmefene or nalmefene were compared by t-test. Although the mean induction times were slightly longer after treatments with U50,488 + nalmefene and nalmefene than after saline (Figure 9.2), there were no significant differences between any of these treatments (all $P>0.05$). Because premature oviposition was not induced by PGF2α in one bird receiving U50,488, the effects of this treatment on oviposition induction time were compared with the other treatments using rank statistics, allocating the highest rank to the oviposition which was not induced (thus, n = 6 for all treatments). Oviposition induction times after treatment with U50,488 were significantly longer compared with saline (Mann-Whitney, w=55, $P<0.01$), but not compared with U50,488 + nalmefene (Mann-Whitney, w=51, $P>0.05$) or nalmefene (Mann-Whitney, w=49, $P>0.05$).

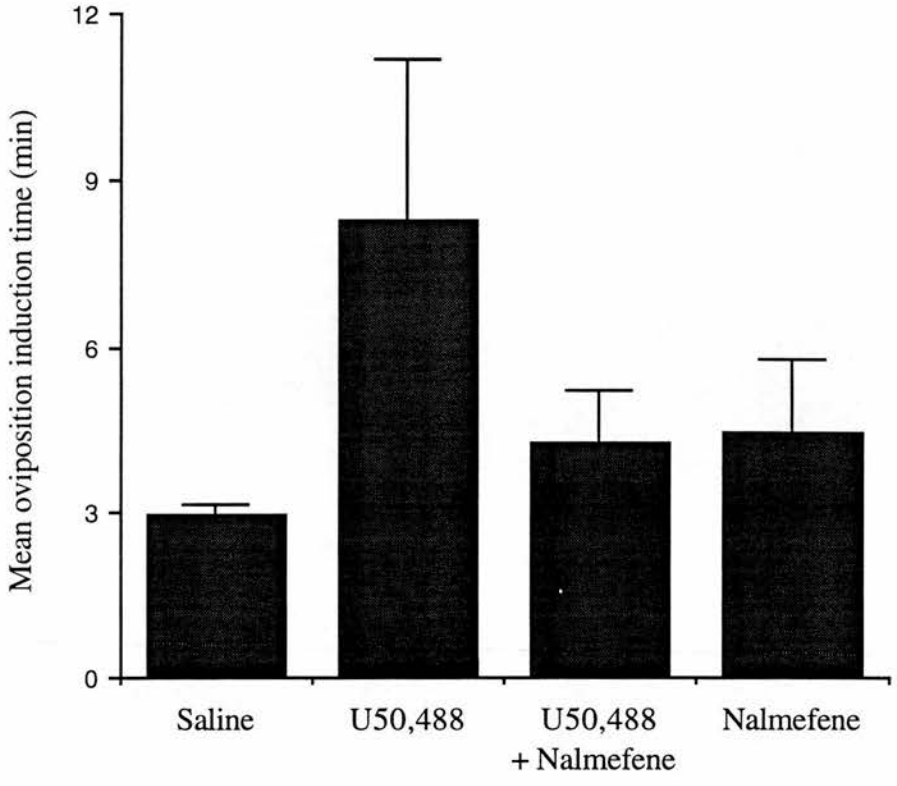



**Figure 9.2** Mean (±SEM) oviposition induction times after injection of 0.5 μg/kg PGF2α in hens treated with either saline, 4 mg/kg U50,488, 4 mg/kg U50,488 + 0.5 mg/kg nalmefene or 0.5 mg/kg nalmefene in Experiment 2 (n = 5 for U50,488 treatment, as one oviposition was not induced, and n = 6 for the other three treatments).

The effects of the four injection treatments on plasma AVT concentrations before PGF2α injection and at the time of induced oviposition are shown in Figure 9.3. There were no significant differences in mean plasma AVT concentrations before PGF2α injection between any pair of treatments (all $P>0.05$). For all treatments, the mean plasma AVT concentrations were significantly greater at the time of induced oviposition compared with those before PGF2α injection (all $P\leq0.05$). Mean plasma AVT concentrations at the time of induced oviposition were similar after treatment with saline, U50,488 + nalmefene or nalmefene and these were higher than that after U50,488 treatment. Mean plasma AVT concentration at the time of induced

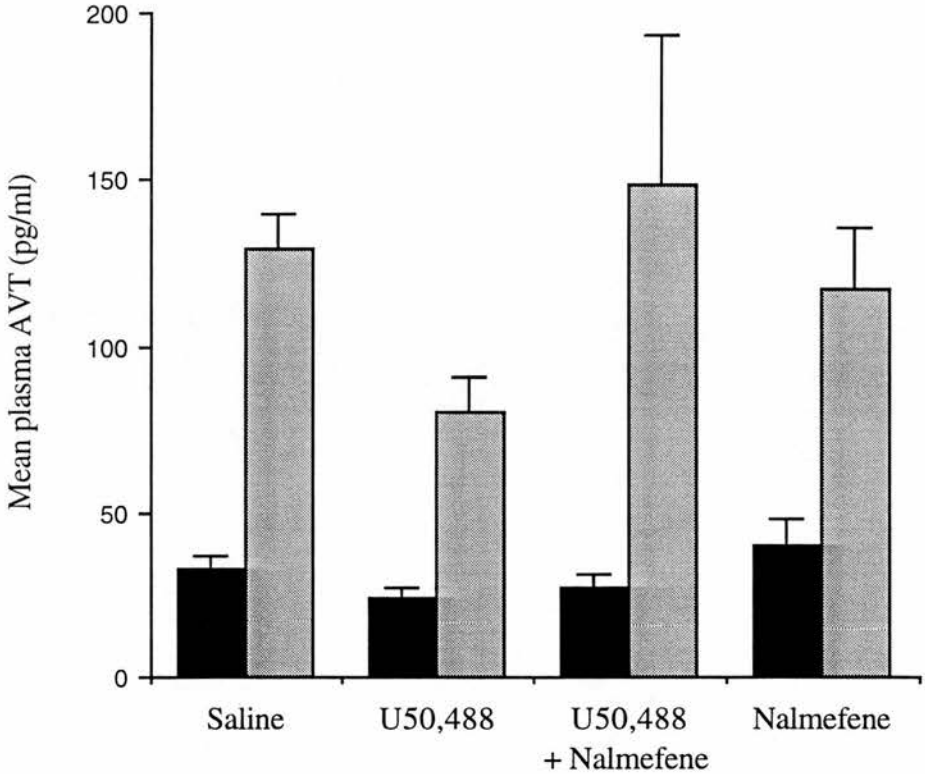



**Figure 9.3** Mean (±SEM) plasma AVT concentrations before injection of PGF2α (dark bars) and at the time of oviposition induced by i.v. exogenous PGF2α (light bars) in hens treated with either saline, 4 mg/kg U50,488, 4 mg/kg U50,488 + 0.5 mg/kg nalmefene or 0.5 mg/kg nalmefene (n = 5 for U50,488 treatment at the time of induced oviposition, as one oviposition was not induced, and n = 6 for all other means)

oviposition was significantly lower in birds receiving U50,488 than in birds receiving saline ($P<0.05$). There were no significant differences in plasma AVT concentration at the time of induced oviposition between any other pair of treatments. Compared with the saline and U50,488 treatments, there was greater variation in the plasma AVT concentrations at the time of induced oviposition for U50,488 + nalmefene and nalmefene treatments, as indicated by the larger SEMs (Figure 9.3).

## 9.4 Discussion

In both Experiments 1 and 2, i.v. PGF2α administered to saline treated hens (controls) induced premature oviposition within a few minutes and, in Experiment 2, it was shown that plasma AVT concentrations were elevated in association with these ovipositions. Saito *et al.* (1987), Shimada *et al.* (1987a) and Murakami *et al.* (1990) have also reported that plasma AVT concentrations are elevated at the time of ovipositions induced prematurely by exogenous PGF2α. The plasma AVT concentrations at the time of induced ovipositions in the present study were similar to those at the time of spontaneous oviposition (Chapter 5) and this is consistent with the findings of Saito *et al.* (1987).

In Experiment 2, oviposition induction times were significantly longer following U50,488 treatment compared with saline treatment. A similar, although non significant, effect was observed in Experiment 1. It was also found (Experiment 2) that mean plasma AVT concentration at the time of induced oviposition was significantly lower after U50,488 injection than after saline injection. In mammals, U50,488 inhibits AVP release in response to dehydration, hyperosmolality and hypovolemia (Oiso *et al.*, 1988), and inhibits release of both oxytocin and AVP vasopressin from isolated neurohypophyses in response to electrical stimulation (Zaho *et al.*, 1988a, Russell *et al.*, 1993). A possible interpretation of the present results is that release of neurohypophysial AVT, stimulated by PGF2α induced uterine contractions, was inhibited by U50,488, and consequently plasma AVT concentrations at induced oviposition were reduced in U50,488 treated hens. As AVT released after PGF2α injection promotes uterine contractions and thus contributes towards the expulsion of the egg (Murakami *et al.*, 1990), the longer oviposition induction times after U50,488 treatment may be a consequence of suppressed AVT release.

In both Experiments 1 and 2, oviposition induction times tended to be longer and more variable after U50,488 + nalmefene and nalmefene treatments compared with saline treatment. In Experiment 2, the plasma AVT concentrations at induced oviposition also tended to be more variable after U50,488 + nalmefene and nalmefene compared with saline. The hypothesis that U50,488 inhibits AVT release in an opioidergic manner predicts that opioid receptor antagonism should prevent this inhibition, and hence that plasma AVT concentrations should not be depressed following U50,488 + nalmefene treatment relative to saline treatment. Mean plasma AVT concentrations at the time of induced ovipositions after treatments with saline, U50,488 + nalmefene and nalmefene were all similar, and were higher than that for

U50,488. However, as a result of the large variability observed after U50,488 + nalmefene and nalmefene treatments, no significant differences in plasma AVT concentrations at induced oviposition were found between these treatments and either saline or U50,488 treatment. Thus, it was not possible to confirm if the reduced AVT release following U50,488 treatment was mediated by opioid receptors.

In addition to its property as a kappa opioid receptor agonist, U50,488 can act in a non opioid manner to directly suppress contractions of smooth muscle (Hayes *et al.*, 1988). Indeed, parturition of rats is delayed by U50,488 acting both to inhibit oxytocin release in an opioidergic manner and to directly suppress uterine contractions in a non opioidergic manner (Douglas *et al.*, 1993). Nalmefene completely prevented the oviposition delaying effect of 4 mg/kg U50,488 in Section 8.6, indicating that U50,488 delays oviposition in an opioidergic manner and not by directly suppressing uterine contractions through non-opioid mechanisms. Hence it is unlikely that U50,488 increased oviposition induction times and reduced AVT release in the present experiment by directly suppressing uterine contractions. Indeed, the fact that all eggs but one were expelled prematurely indicates that uterine contractions, the stimulus for AVT release, were present.

There were no differences between any of the treatments in plasma AVT concentrations before PGF2α injection. The lack of an effect of nalmefene may indicate that basal AVT release is not regulated by endogenous opioids, and the lack of an effect of U50,488 may indicate that basal AVT release is not inhibited by opioids acting via kappa receptors. Alternatively, treatments with U50,488 and nalmefene may affect basal AVT release but these effects were not evident due to the short time between treatment and blood sampling (mean = 13 min). Oiso *et al.* (1988) found that basal plasma AVP concentrations were reduced in rats 60 min after receiving 5 mg/kg U50,488 subcutaneously.

In mammals, opioids can act in the hypothalamus (mu and kappa receptors) and in the neurohypophysis (kappa receptors only) to inhibit the release of neurohypophysial hormones (see Bicknell (1985) for a review). Opioids may also act on spinal sites to inhibit reflex oxytocin release (Wright *et al.*, 1985). In hens, only one type of opioid receptor (not yet identified) has been found in the neurohypophysis and it appears that this receptor is located on the terminals of mesotocin but not AVT neurones (Martin *et al.*, 1992; Kawashima *et al.*, 1995; also see General Introduction). As opioid receptors have not been associated with the terminals of AVT neurones, it is unlikely that U50,488 inhibits AVT release by acting on the neurohypophysis. In Chapters 7 and 8, both stress and U50,488 delayed oviposition in a nalmefene reversible manner, and it was hypothesised that these delays were due to opioidergic

inhibition of neurohypophysial AVT release. The finding that U50,488 inhibits the release of AVT associated with PGF2α-induced premature ovipositions supports the hypothesis that opioids can inhibit AVT release and that such a mechanism may underlie oviposition delays.

Of the 32 hens selected for treatment in Experiment 1, 7 had to be excluded from the analyses of results because their egg sequences ended within three days of treatment or because no egg was carried on the day of treatment. By contrast, none of the 24 hens selected for treatment in Experiment 2 were excluded from the analysis. The hens in Experiment 1 (52-55 weeks old) were older than those in Experiment 2 (27-32 weeks old) and this may account for the above difference between the Experiments. As older birds tend to lay shorter sequences (i.e. fewer eggs per sequence) than younger birds which are at the peak of egg production (Lillpers & Wilhelmson, 1993a), the incidence of egg sequences ending within three days of treatment will tend to be greater for older birds. As the frequency of internal ovulations increases with age (Lillpers & Wilhelmson, 1993a), and as internally ovulated eggs probably account for instances where selected birds did not carry hard-shelled eggs on the day of treatment, excluding hens from analysis for this reason is likely to occur more often in experiments with older birds.

To summarise, U50,488 delayed the premature induction of oviposition following PGF2α injection and this was associated with reduced AVT release. This finding supports the hypothesis that opioids can act to inhibit AVT release and that such a mechanism may underlie oviposition delays.

# CHAPTER 10: **Use of Headflick Frequency as a Behavioural Indicator of Hens' Opioid Status as Affected by Social Stress**

## 10.1 Introduction

It is hypothesised that endogenous opioids released in response to stress are involved in the mechanism underlying oviposition delays, and that they act by inhibiting release of AVT from the posterior pituitary (see General Introduction). To evaluate this hypothesis it is important to consider the effect of the social stress used in this study on the opioid status of the hens. In mammals, concentrations of endogenous opioids in both brain and plasma are elevated in response to various stressors (e.g. Madden *et al.*, 1977; Rodway *et al.*, 1993). As opioidergic inhibition of neurohypophysial hormone release can be either central or peripheral (Bicknell, 1985), stress-induced oviposition delays could be due to elevated opioid activity in either brain or plasma.

Although the concentrations of various opioids in mammalian plasma and brain tissues can be measured by assay techniques, these are unsuitable for measuring the effect of social stress on release of endogenous opioids in hens. Firstly, opioid assays have not been validated for use in hens. Secondly, it is not known which endogenous opioids might influence oviposition. Thirdly, important stress-related changes in the opioid content of various brain regions may be small, so there are practical difficulties in their measurement (Akil *et al.*, 1988).

In addition to direct measurement of opioid levels, opioid status of mammals can be investigated using behavioural indices which typically reflect pain perception. For example, in rats the latency from a locally applied painful stimulus to tail-flicking correlates with brain opioid activity (Madden *et al.*, 1977; Kulling *et al.*, 1989). Behavioural tests of analgesia have been used to demonstrate release of endogenous opioids in response to various environmental stimuli, such as stress, novelty and feeding (Rodgers & Randall, 1989). Environmentally and pharmacologically induced analgesia in chicks have been studied by measuring behavioural responses to painful stimuli, such as pressure applied to foot (Schneider, 1961), high temperature applied to foot or plucked area of skin (Fan *et al.*, 1981; Sufka & Hughes, 1990; Sufka *et al.*, 1994), and electric shock applied to wing or leg (Bardo & Hughes, 1978; Rager & Gallup, 1986). These tests have not been used for measuring analgesia in adult hens. Hence, the following study investigated the possible use of hens' headflick response to alerting stimuli, a putative measure of

arousal or responsiveness, as a behavioural indicator of opioid status. From an ethical perspective, measures of headflicking are preferable because they do not involve nociceptive stimulation, as used in tests of analgesia.

Headflicks, also described as headshakes, are distinctive and rapid lateral movements of the hen's head (Kruijit, 1964). The frequency of headflicking is elevated in response to alerting stimuli, such as the presence of an observer or exposure to noise, and is thought to indicate the level of arousal of the hen (Hughes, 1983; Savory *et al.*, 1993a). Arousal has been described as "a hypothetical physiological variable which correlates with overall behavioural responsiveness and activity, not necessarily always in terms of overt physical behaviour, but also as covert nervous system activity" (Delius, 1970 p.175). Savory and Hughes (1988) found that treatment with the non-specific opioid antagonist nalmefene influences the frequency of headflicking by laying hens in a dose dependant manner. A low dose (0.2 mg/kg) and high doses (0.8 and 1.6 mg/kg) of nalmefene did not alter headflick frequency while an intermediate dose (0.4 mg/kg) elevated it. One interpretation was that endogenous opioids have de-arousing properties and that, by blocking opioid receptors and so removing a basal opioid influence, nalmefene increases the level of arousal and consequently the frequency of headflicking. Low levels of headflicking following 0.8 and 1.6 mg/kg nalmefene were thought to represent reduced arousal due to sedative effects of this drug at these higher doses. Nalmefene has similar dose dependant effects on spontaneous headflick frequency in broiler breeder fowls (Savory *et al.*, 1993a). Thus, the frequency of headflicking may be related positively to the level of arousal and negatively to opioid status in such a way that increases in endogenous opioid activity are expected to cause de-arousal and so reduce headflick frequency.

The parent stock of broiler chickens, (broiler breeders) are maintained on a restricted feeding regime to control their body weight, and as a result are chronically hungry (Savory *et al.*, 1993b). Savory *et al.* (1993a) reared broiler breeders on 3 levels of food intake: restricted rations (R), 2 x restricted rations (2R) and *ad lib.* food access (AL; food intake was about 3 x restricted rations). At 21 weeks of age headflick frequency during noise was significantly greater for the AL than the R birds, with 2R birds being intermediate. Although studies of opioid-like immunoreactivity in brains from these birds were inconclusive (Savory *et al.*, 1993a), there were significant increases in delta and mu opioid receptor densities in R birds (compared with 2R and AL) in certain areas of the striatum (Savory & Stewart, in preparation). As opioid status is influenced by the density of opioid receptors in addition to the amount of opioid release, these findings are consistent

with the hypothesis that headflick frequency reflects opioid status.

It was predicted that exposing hens to social stress would cause the release of endogenous opioids and these would, through their de-arousing properties, reduce the frequency of headflicking. In Experiment 1, it is shown that social stress causes a reduction of hens' headflick frequency relative to control treatment. Experiment 2 examined, through the use of the non-selective opioid receptor antagonist nalmefene (see Section 1.6.1 for outline of pharmacology), the role of endogenous opioids in the stress-related reduction in headflick frequency observed in Experiment 1. However, in Experiment 2, social stress did not depress headflick frequency as it had in Experiment 1, and so the possibility that the saline injection procedure, present in Experiment 2 but not Experiment 1, influenced headflick frequency was tested in Experiment 3.

## 10.2 Experiment 1: Effect of Social Stress on Headflick Frequency

The aim of this experiment was to test the hypothesis that social stress would reduce the frequency of headflicking by hens.

### 10.2.1 Materials and Methods

The headflick responses to alerting stimuli of 20 hens aged 53 weeks were tested following both stress and control treatments. Hens were allocated randomly to receive either the stress treatment on the first of three consecutive days and the control treatment on the second and third or vice versa (n = 10 each). The third day of testing was included to allow an analysis which included possible treatment carry-over effects. Hens were either exposed to social stress for a period of 21-59 min (mean = 38 min) before testing or left undisturbed until testing (control). Testing involved isolating each hen in a cage (30 cm wide, 45 cm deep, 37-46 cm high), with solid sides and a sloping mesh floor in the middle tier of a battery cage system contained in an unfamiliar room, where it was exposed to a standard 4 min of varying noise (music) stimulation. The number of headflicks during this period was recorded on a tally counter by an observer sitting opposite and in full view of the hen. The noise was expected to increase the frequency of spontaneous headflicking by acting as an alerting stimulus (Savory *et al.*, 1993a), so improving the ability to detect differences in arousal between the two treatments. The test conditions also included a novel environment and the presence of an observer, both of which elevate

the frequency of headflicking (Hughes, 1983). Hens were tested alternately from control and social stress treatments, with the order and time of testing birds being the same on both days. As the headflick response may show a diurnal rhythm (Preston, 1984), it was important that observations on each bird were made at a similar time each day. All birds were tested in the afternoon and, as it is not known if pre-laying behaviour or oviposition affect headflick frequency, birds which had not already laid were palpated to confirm that they did not carry a hard-shelled egg.

### 10.2.2 Results

Figure 10.1 shows the effects of stress and control treatments on days 1 and 2 (the first exposure to each) on headflicking. It can be seen that there was much inter-bird variation in the amount of headflicking. Initially a non-parametric analysis

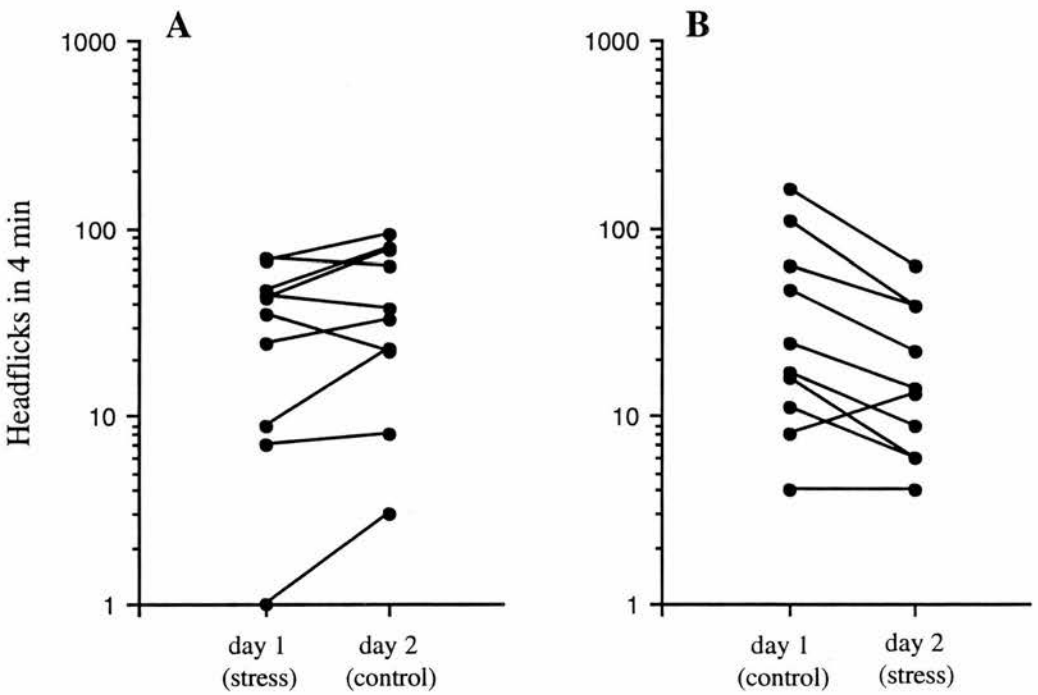



**Figure 10.1** The effect of stress and control treatments on the number of headflicks during 4 min of exposure to novel noise in an unfamiliar room for hens receiving either stress on day 1 and a control treatment on day 2 (A) or vice versa (B).

was conducted. Considering only days 1 and 2; 15 of the 20 birds showed more headflicking after control treatment than after stress, 4 birds headflicked more after stress treatment than after control treatment and 1 bird showed no difference. Thus, social stress significantly lowers the frequency of headflicking in response to the novel stimuli of the test conditions (sign test, $P<0.05$).

To test for possible day, bird and treatment carry-over effects, the data were log transformed to normalise their distribution and produce a more homogeneous scatter of variance, thus permitting the use of parametric statistics. There were significant effects of treatment ($F=25.01$; 1,37 df; $P<0.001$ by ANOVA) and bird ($F=43.41$; 18,37 df; $P<0.001$ by ANOVA) but not day of testing ($F=0.77$; 2,37 df; $P>0.05$ by ANOVA) on the number of headflicks. A test for carry-over effects found no significant influence of previous treatment on headflick response ($X^2=0.36$, 1 df, $P>0.05$).

Back-transformed mean number of headflicks in 4 min was 27.2 for the control treatment and 18.1 for the stress treatment (corrected for bird and potential day effects by GLM).

## 10.3 Experiment 2: Does Nalmefene Prevent the Reduction of Headflick Frequency Associated with Social Stress?

In Experiment 1, the frequency of headflicking in response to alerting stimuli was significantly lower following exposure to social stress compared with no stress. One interpretation of this finding is that social stress causes the release of endogenous opioids which, through their de-arousing properties, reduce the frequency of headflicking. If the stress-related reduction of headflick frequency is mediated by endogenous opioid release then this reduction should be prevented by treatment with an opioid receptor antagonist. Experiment 2 investigated the effect of nalmefene, a non-selective opioid receptor antagonist, on the stress-related reduction in headflick frequency.

### 10.3.1 Materials and Methods

The headflick responses to alerting stimuli of 16 hens aged 30 weeks were tested following each of four treatments: saline injection, saline injection then social stress, 0.5 mg/kg nalmefene injection, 0.5 mg/kg nalmefene injection then social stress. Each bird was exposed to a different treatment on each of four consecutive

days according to the following design. Latin squares balanced for carry-over effects and for n treatments have n different treatment orders, such that each treatment follows each other treatment only once and each treatment occurs once each day (John, 1971). For four treatments there are two possible Latin squares balanced for carry-over effect and thus eight different treatment orders. Two birds were allocated to each of these possible treatment orders (i.e. two of each Latin square). Thus, each treatment followed each other treatment four times, each treatment occurred four times each day and each bird received each treatment once. The order of testing the 16 birds was randomised for day 1, then this order was maintained for the following days so that individuals could be tested at the same time each day. As in Experiment 1, all treatments were in the afternoon and birds were palpated beforehand if it was not known if they carried a hard-shelled egg.

Nalmefene (6-desoxy-6-methylene naltrexone, Key Pharmaceuticals, Miami) was dissolved in saline (1.0 mg/ml) and stored at -18$^{o}$C until required. Following 0.5 ml/kg i.m. injection of 0.9% saline or nalmefene, hens were either exposed to social stress or returned to their home cages until headflick frequency testing. An additional 24 hens, also 30 weeks of age, were used to make up the stress groups (8 groups x 3 hens), the composition of which varied each day so that all four hens in each group were always strangers. The number of headflicks during 4 min exposure to alerting stimuli was tested 36-63 min (mean = 53 min) after injection by the procedure described in Experiment 1 but with the following modification. As the cage used for testing in Experiment 1 was no longer available, during test periods hens were placed in a single cage (40 cm wide, 45 cm deep, 45 cm high) with mesh sides and a level solid floor.

The data were log transformed to provide a more normal distribution and homogeneity of variance. To allow for zero values, 1 was added to each score before transformation.

### 10.3.2 Results

As the carry-over on day 1 was the same for all birds (i.e. no previous treatment), carry-over and day effects were not independent and so could not be included in the same GLM analysis. As there was no significant influence of previous treatment on the number of headflicks ($F=1.34$; 3,39 df; $P>0.05$), carry over was excluded from the analysis. There were significant effects of bird ($F=29.95$; 15,42 df; $P<0.001$) and treatment ($F=7.07$; 3,42 df; $P<0.001$), but not of day ($F=2.3$; 3,42 df; $P>0.05$) on the number of headflicks. To clarify the treatment effects, the

treatment means (corrected for bird and potential day effects by GLM) were compared by t-test. The treatment means with 95% confidence limits and the results of the treatment comparisons are shown in Figure 10.2. Nalmefene elevated the number of headflicks relative to the control (saline) treatment, both with and without social stress. Social stress did not affect the number of headflicks, following either saline or nalmefene injections.

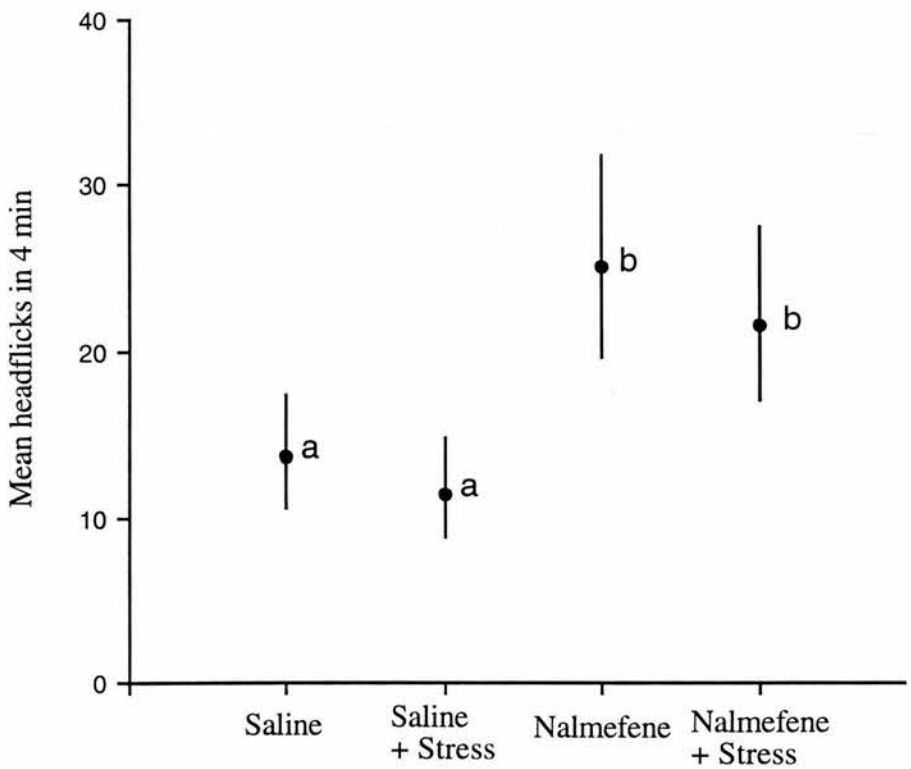



**Figure 10.2** Back-transformed mean number of headflicks in 4 min with 95% confidence limits (corrected for bird and potential day effects by GLM) for each of 4 treatments: saline injection, saline injection plus social stress, 0.5 mg/kg nalmefene injection, 0.5 mg/kg nalmefene injection plus social stress (n = 16 each). Means with different letters differ significantly ($P<0.05$).

## 10.4 Experiment 3: Do Intramuscular Saline Injections Affect Headflick Frequency?

Although hens headflicked less frequently in response to alerting stimuli after social stress than after no stress in Experiment 1, this effect of stress was not found in Experiment 2. One possible explanation for this difference is that the i.m. injection procedure, present in Experiment 2 but not in Experiment 1, was stressful or painful causing endogenous opioid release and so reducing headflick frequency in all treatments (i.e. in birds exposed to stress and in those which were not). The fact that mean headflick frequency for the control treatment in Experiment 2 (stress + saline injection; 13.6 headflicks in 4 min) was lower than that in Experiment 1 (stress alone; 27.2 headflicks in 4 min) is consistent with this suggestion. To test the possibility that the i.m. injection procedure may reduce headflick frequency, Experiment 3 compared the effects of saline injection and no injection on the frequency of headflicking.

### 10.4.1 Materials and Methods

The headflick response of 12 hens aged 31 weeks was tested following both saline injection and control treatments. The birds were randomly allocated to receive either the injection treatment on the first of two consecutive days and the control treatment on the second or vice versa (n = 6 each). Birds were injected i.m. with 0.5 ml/kg saline 36-55 min (mean = 45 min) before testing or left undisturbed until testing (controls). All treatments were in the afternoon and birds were palpated beforehand if it was not known if they carried a hard shelled egg. The number of headflicks during 4 min exposure to varying noise was tested as in Experiment 2.

The data were log transformed to provide a more normal distribution and homogeneity of variance. To allow for zero values, 1 was added to each score before transformation.

### 10.4.2 Results

There was a significant effect of bird ($F=4.22$; 11,10 df; $P<0.05$ by GLM) but not of treatment ($F=3.29$; 1,10 df; $P>0.05$ by GLM) or day ($F=0.63$; 1,10 df; $P>0.05$ by GLM) on the number of headflicks. For both treatments the mean number of headflicks (corrected for bird and potential day effects by GLM) with 95% confidence limits are shown in Figure 10.3.

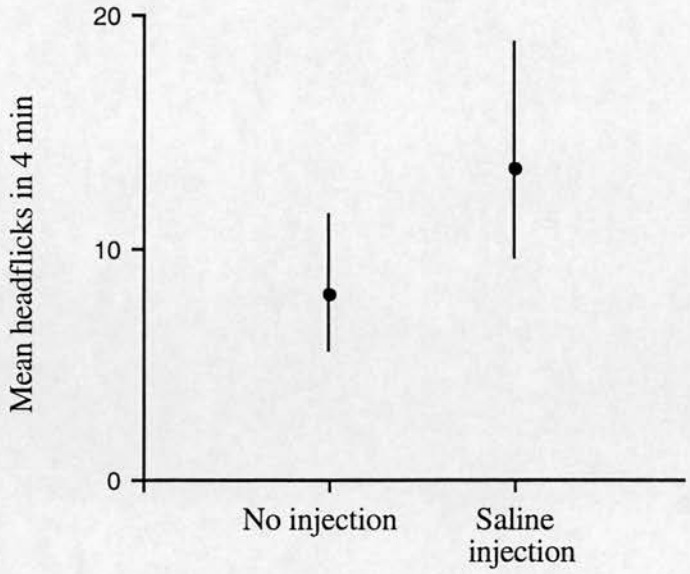



**Figure 10.3** Back-transformed mean number of headflicks in 4 min with 95% confidence limits (corrected for bird and potential day effects by GLM) following saline injection or no treatment (n = 12 each).

## 10.5 Discussion

As headflick frequency may reflect the level of arousal and is elevated following treatment with an opioid antagonist it is thought that endogenous opioids may have de-arousing properties (Hughes, 1983; Savory & Hughes, 1988; Savory *et al.*, 1993a). In Experiment 1, the reduction of hens' headflick frequencies caused by social stress may reflect the release of endogenous opioids. This explanation is consistent with reports that various stressors, including social stress, cause opioid release in mammals (e.g. Madden *et al.*, 1977; Rodway *et al.*, 1993). If the stress-related reduction of hens' headflick frequency is indeed opioid mediated, then this finding supports the hypothesis that opioids released in response to social stress may be involved in the mechanism underlying oviposition delays.

To confirm that the stress-related reduction of headflick frequency observed in Experiment 1 reflects release of endogenous opioids, Experiment 2 was designed to test whether antagonism of opioid receptors could prevent this response. However, in Experiment 2, which investigated the effect of nalmefene on stress-related reduction of headflick frequency, social stress did not reduce the frequency of

headflicking. Additionally, mean headflick frequency for the control treatment in Experiment 2 (stress + saline injection; 13.6 headflicks in 4 min) was lower than that in Experiment 1 (stress alone; 27.2 headflicks in 4 min). It is not clear why stress depressed headflick frequency in Experiment 1 but not in Experiment 2, why headflick frequency varied between the control treatments of these two experiments and if these two differences are related. There were some differences between these experiments which may be important. First, it is possible that the injection procedure, present in Experiment 2 but not in Experiment 1, was itself stressful or painful and so caused the release of endogenous opioids which depressed headflick frequency in all hens (both those exposed to stress and the controls) in such a way that the effects of stress were masked. However, as there was no effect of i.m saline injections on headflick frequency in Experiment 3, it is unlikely that this procedure accounts for the lack of effect of stress in Experiment 2. Second, the test conditions in which headflick frequencies were measured differed slightly between Experiments 1 and 2 and this may have influenced the headflick response. Although it is not clear how headflicking might be affected by different test conditions, the results of behavioural tests of analgesia in mammals can be greatly influenced by subtle changes in test procedure (Amit & Galina, 1986). For example, Bodnar *et al.* (1984) found that by varying the temperature applied to the tail in the rat tail-flick assay, opposite results to the same treatments could be generated (i.e. analgesia vs. algesia). Third, the duration of social stress prior to headflick frequency testing was longer in Experiment 2 (mean = 53 min) than in Experiment 1 (mean = 38 min) and perhaps the lack of effect of stress in Experiment 2 reflects a time related release of endogenous opioids during social stress. In mammals the presence of opioid based stress-induced analgesia is sometimes related to the duration of the stress (see Rodgers & Randall, 1988). Fourth, the hens in Experiment 1 (53 weeks old) were older than those in Experiment 2 (30 weeks old) and, although it is not clear how, age may influence the effects of stress on headflick frequency.

In Experiment 2, 0.5 mg/kg nalmefene significantly increased, relative to treatment with saline, headflick frequency following either social stress or no stress. Savory and Hughes (1988) and Savory *et al.* (1993a) found that similar doses of nalmefene also elevated headflick frequency of immature hens of an egg-laying strain and of broiler breeders, and suggested that this reflects increased arousal due to antagonism of endogenous opioids which have de-arousing properties. The present study demonstrates that opioid receptor antagonism also increases headflick frequency in mature laying hens.

A further finding from these experiments is that there is much interbird

variation in the frequency of headflicking in response to alerting stimuli and this is consistent with the findings of Hughes (1983). It is therefore desirable, when investigating the effects of different treatments on headflick frequency, that experiments are designed in such a way that each hen acts as its own control.

Sherwin *et al.* (1993) reported that food deprivation followed by transportation for 6 h increased the latency to the first headflick following presentation of an alerting stimulus to broiler chickens. As longer latencies to first headflick are likely to reflect reduced headflick frequencies, their results suggest that food deprivation followed by transportation may reduce headflick frequency. Thus, both social stress and transportation following food deprivation reduce headflick frequency, and it is hypothesised that this reflects the release of endogenous opioids with de-arousing properties.

To validate the use of headflick frequency as a measure of opioid status further experiments are required. It still needs to be confirmed, through treatment with an opioid antagonist, that the stress-related reduction in headflick frequency observed in Experiment 1 is indeed opioid mediated. If low headflick frequencies reflect endogenous opioid release then it would be predicted that headflick frequency would be reduced by injection of exogenous opioid agonists. However, in such experiments it may be difficult to separate opioid agonist effects from potential sedative effects. The effects of other environmental stimuli which are thought to alter hens' opioid status on headflick frequency could be investigated. For example, as feeding is thought to cause the release of endogenous opioids (Savory *et al.*, 1989) and reduces in a naloxone reversible manner the performance of pain related behaviours associated with injection of sodium urate into the ankle joint (Wylie, 1995), it is predicted that feeding will act to reduce headflick frequency. As tests of analgesia are often used as measures of opioid status in mammals (Rodgers & Randall, 1988), it would be interesting to compare the effects of various environmental stimuli on both headflick frequency and analgesia in hens. Although there are no established methods for testing analgesia in adult hens, tests such as the hot plate test are frequently used in chicks (e.g. Hughes, 1990). As chicks show headflicking in response to alerting stimuli (personal observations), they could be used in a study comparing headflicking and analgesia.

As arousal is a higher brain process and endogenous opioids do not cross the blood brain barrier, it is likely that opioids influencing headflick frequency originate and act centrally. However, as with tests of analgesia, changes in headflick frequency provide no information on which opioids are released in response to environmental stimuli.

In conclusion, although social stress reduced headflick frequency in Experiment 1, no such effect was found in Experiment 2 and so the role of opioids in stress-related reduction of headflick frequency could not be tested. However, in Experiment 2 the opioid antagonist nalmefene did increase headflick frequency, indicating normal inhibitory opioid tone on this activity. The results of Experiment 1 could still be consistent with the hypothesis that opioid release is involved in the mechanism underlying oviposition delays induced by social stress.

# CHAPTER 11: **Does Propranolol Reduce the Duration of Stress-Induced Oviposition Delays?**

## 11.1 Introduction

The release of adrenaline, from the adrenal glands and into the circulation, is involved in mediating hens immediate responses to stressors (Freeman, 1976; Harvey *et al.*, 1984). Exogenous adrenaline acts via beta adrenergic receptors to cause relaxation of the hen uterus *in vitro* (Sykes, 1955; Crossley *et al.*, 1980) and delays oviposition *in vivo* (Sykes, 1955; Crossley, 1983) (see Section 1.7 for a review). It has been hypothesised that stress delays oviposition by causing an adrenergic suppression of uterine contractions (Sykes, 1955; Weiss & Sturkie, 1952; Hughes & Black, 1976; Crossley, 1983). If this is correct then treatment with propranolol, a beta adrenergic receptor blocker, should reduce the duration of these delays. The aim of the following study was to test this prediction. Effects of propranolol on environmentally induced oviposition delays have not been reported previously.

In the first experiment (Section 11.2) propranolol prevented stress-induced oviposition delays. However, it also appeared to induce premature oviposition of control hens and a second experiment (Section 11.3) was designed to further investigate this.

## 11.2 Does Treatment with 10 mg/kg Propranolol Reduce the Duration of Stress-Induced Oviposition Delays?

The aim of this experiment was to investigate the effects of propranolol on the oviposition times of both undisturbed hens and those exposed to social stress. In particular, would propranolol reduce the duration of stress-induced oviposition delays?

### 11.2 Materials and Methods

From 35 naive hens aged 24 weeks, a total of 31 whose next oviposition times could be predicted were selected over two days. Selected hens were randomly allocated to receive either 0.9% saline injection (n = 7), 0.9% saline injection followed by social stress (n = 8), 10 mg/kg propranolol injection followed by social stress (n = 8) or 10 mg/kg propranolol injection (n = 8). A lower dose of propranolol (5 mg/kg)

suppresses object pecking in broiler breeder fowls, but only for up to 1 h, without apparent sedation (Savory & Kostal, 1994). In order to provide a longer lasting effect 10 mg/kg was used in the present experiment, a dose which influences the respiratory quotient of cockerels for several hours without affecting daily activity level (MacLeod & Watson, 1992).

Propranolol (±propranolol hydrochloride; Research Biochemicals Incorporated, Natick, MA) was dissolved in 1M acetic acid then diluted with 0.9% saline to a concentration of 20 mg/ml. Both the propranolol solution and the saline for control injections were adjusted to pH 6.0 by titration with sodium hydroxide and acetic acid. Injections were 0.5 ml/kg i.m., 40 min before predicted oviposition time.

Immediately after injection, each hen due to receive stress was placed in a group cage together with three unfamiliar hens. These unfamiliar hens were from an additional stock of 57-week-old hens, housed individually in bottom tier battery cages. For all hens in a group, exposure to social stress was ended by returning them to their original cages once the injected hen laid, or after a period of 4 h if this did not occur. Hens that were not due to be stressed were returned to their original cages immediately after injection.

### 11.2.2 Results

Two hens (1 x propranolol + stress treatment, 1 x propranolol treatment) were excluded from the following analysis as they laid before they were due to be injected (i.e. more than 40 min before predicted oviposition time).

Four hens, all receiving saline + stress, did not lay during stress and were considered to have oviposition delays of 180 min for the following analysis. Mean oviposition delay duration for each of the four treatments and the statistical comparisons between these are shown in Figure 11.1. Social stress induced oviposition delays and propranolol significantly reduced the duration of these. Indeed, there was no significant difference in delay duration between hens receiving saline but not stress and those receiving propranolol and exposed to stress. For hens not exposed to social stress, propranolol caused ovipositions to occur earlier than expected. It should be noted that injections were 40 min before predicted oviposition time and so some hens receiving propranolol but not stress laid shortly (range = 8-48 min, mean = 23.1 min) after injection.

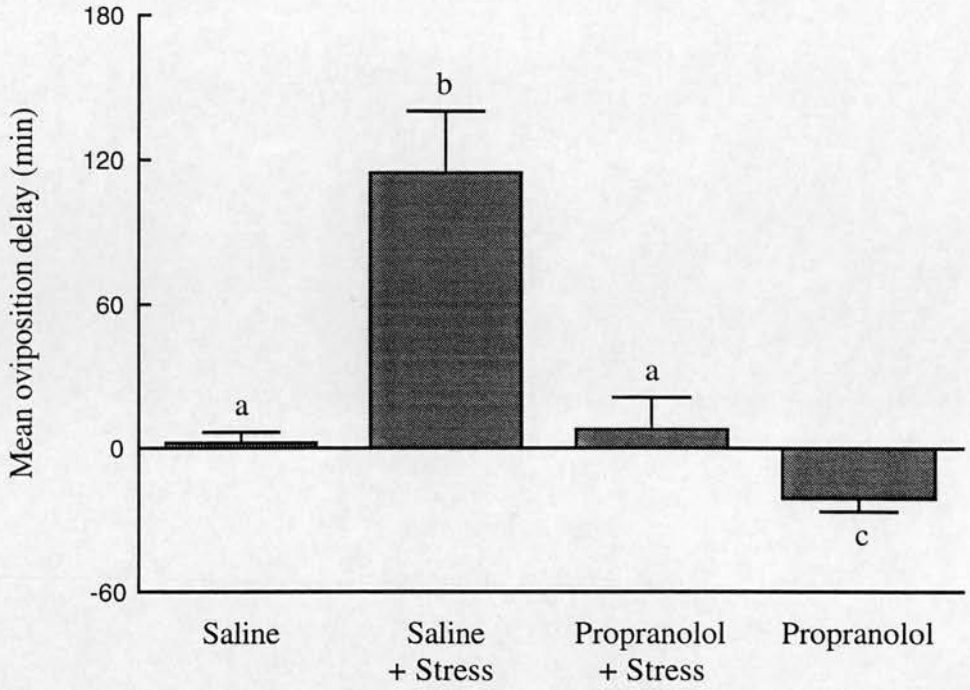



**Figure 11.1** Mean (±SEM) duration of oviposition delay for hens receiving either saline injection, saline injection + social stress, 10 mg/kg propranolol injection + social stress or 10 mg/kg propranolol injection (n = 7-8 for each). Means with different letters differ significantly ($P<0.05$ by Mann-Whitney).

## 11.3 Does Propranolol Affect Oviposition Time of Undisturbed Hens?

In Section 11.2, hens receiving propranolol injection but not social stress laid earlier than expected and this effect was significant when compared with the control treatment (saline injection). Presumably propranolol injection could act in either of two ways to cause this effect. First, it may act to initiate oviposition prematurely. As propranolol blocks beta adrenergic receptors, circulating adrenaline will act on the uterus via alpha receptors only and so will cause contractions (Crossley, 1980). Possibly stress associated with the injection procedure causes adrenaline release which then initiates oviposition by inducing contractions. Indeed, it has been shown that propranolol induces contractions of the hen uterus *in vivo* (Verma & Walker, 1974; Murayama *et al.*, 1980). Propranolol is also reported to terminate pregnancy in

women by acting as a labour inducing agent (Roy *et al.*, 1985). If this hypothesis is true then oviposition time may be related to injection time (i.e. oviposition occurs a short time after injection). Second, propranolol may act to hasten the normal oviposition mechanisms, in which case oviposition time may be related to expected oviposition time rather than to injection time. Possibly an inhibitory adrenergic mechanism modulates oviposition time in unstressed hens and propranolol allows oviposition to occur earlier than normal by blocking this. Alternatively, the effect of propranolol on oviposition time in Section 11.2 might not be a real one and may have occurred through random chance.

The aim of the following experiment was to re-examine the effect of propranolol on the oviposition times of undisturbed hens. So that any premature ovipositions could be more easily associated with either the injection time or the expected oviposition time, hens were injected 2 h before predicted oviposition time.

## 11.3 Materials and Methods

From 35 stock hens aged 26 weeks, 16 whose next oviposition times could be predicted were selected and randomly allocated to receive an injection of either 0.9% saline or 10 mg/kg propranolol (n = 8 each). Propranolol and saline were prepared and injected as described in Section 11.2.1 with the exception that injections took place 2.0 h before predicted oviposition time. All hens were replaced in their original cages immediately after injection.

### 11.3.2 Results

Mean Δ oviposition times after injection of either saline or propranolol are shown in Table 11.1. There was no significant difference between the two treatments (t-test; t=1.64, 14df, $P>0.05$).

| Injection | Mean Δ oviposition time (min) |
|---|---|
| Saline | -10.4 (5.7) |
| Propranolol | -24.6 (6.6) |

**Table 11.1** Mean (± SEM) Δ oviposition time for hens receiving either saline or 10 mg/kg propranolol (n = 8 each).

## 11.4 Discussion

The major finding from Section 11.2 is that propranolol, a beta adrenergic receptor antagonist, reduces the duration of oviposition delays induced by social stress. Indeed, relative to control hens (saline injection and no social stress), no oviposition delay was evident in hens exposed to social stress and receiving propranolol. This supports the hypothesis that stress acts via adrenergic pathways to induce oviposition delays (Weiss & Sturke, 1952; Sykes, 1955; Hughes & Black, 1976; Crossley, 1983). The proportions of alpha and beta adrenergic receptors varies along the length of the oviduct such that adrenergic stimulation causes relaxation of the uterus and contraction of the uterovaginal sphincter (Verma *et al.*, 1977, Crossley *et al.*, 1980; Wechsung & Houvenaghel, 1987). It is therefore possible that adrenaline released in response to stress delays oviposition both by promoting contractions of the uterovaginal sphincter (alpha receptors) and by suppressing contractions of the uterus (beta receptors). Propranolol may prevent these delays by blocking the beta receptor mediated suppression of uterine contractions.

There are now several lines of evidence supporting the hypothesis that a direct adrenergic suppression of uterine contractions may be involved in delaying oviposition during environmental stress. Various stressors cause the release of adrenaline from the adrenal medulla in hens (Freeman, 1976; Harvey *et al.*, 1984). Oviposition delays can be induced by exogenous adrenaline (Sykes, 1955; Crossley, 1983; Hughes & Gilbert, 1984) and these can be prevented by propranolol (Crossley, 1983). Stimulation of adrenergic receptors cases relaxation of uterine muscle, both *in vitro* and *in vivo* (Verma & Walker, 1974; Verma *et al.*, 1977; Crossley, 1980; Wechsung & Houvenaghel, 1987). The present study now demonstrates that propranolol can prevent environmental stress from inducing oviposition delays.

Propranolol acts to reduce anxiety in man (Bowman & Rand, 1980) and presumably may have similar tranquillising effects in hens and these may contribute towards the reduction in stress-induced oviposition delay duration observed in the present study. Thus, relocation from individual to group cages may have been less stressful for hens receiving propranolol (due to its tranquillising effects), so shortening oviposition delays, than for hens receiving saline.

Although appropriate control treatments, such as no injection, were not present, saline injections alone did not appear to induce oviposition delays in either of the experiments reported here. By contrast, Crossley (1983) found that subcutaneous injections of saline at 4 ml/hen delayed oviposition for about 4.5 h. Possibly the larger volume injected and subcutaneous route used in that experiment was more

stressful than the injection procedure in the present experiments (intramuscular injection of saline at approximately 1 ml/hen).

In Section 11.2, hens receiving propranolol injection but not social stress laid earlier than expected and this effect was significant when compared with the control treatment. Two possible explanations were suggested (see Section 11.2 Introduction). Briefly, propranolol might initiate the oviposition process prematurely or may allow oviposition to occur earlier than normal, possibly by blocking an endogenous adrenergic inhibition of oviposition. In Section 11.3, hens receiving propranolol 2 h before predicted oviposition time also laid earlier than expected, and by a similar amount to those in Section 11.2. However, in Section 11.3 this effect was not significant when compared with the control treatment. As eggs were not expelled shortly after propranolol injections given 2 h before predicted oviposition time, it seems unlikely that oviposition is initiated by propranolol. That there was a wide range of times from propranolol injection until oviposition in Section 11.2 (8-48 min) is also consistent with the conclusion that this treatment does not induce premature oviposition. Instead, propranolol presumably allows oviposition to occur slightly earlier than normal. This effect on oviposition was not reported when propranolol was included in hens' food (Crossley, 1983).

The mechanism by which propranolol causes oviposition to occur earlier than expected is not known. When isolated uterine muscle is pre-treated with propranolol adrenaline is only able to act via alpha receptors and so causes contraction (Crossley *et al.*, 1980). Perhaps the onset of oviposition is stressful, causing adrenaline release which, in the presence of propranolol, causes uterine contractions which expel the egg earlier than normal. Another possibility is that there may be an inhibitory adrenergic pathway which modulates the oviposition time of undisturbed hens and propranolol blocks this and so allows oviposition to occur earlier than normal. Propranolol facilitates electrically stimulated release of oxytocin in lactating rats (Russell *et al.*, 1993). Perhaps propranolol similarly facilitates AVT release in hens and this contributes towards its oviposition advancing effect.

In conclusion, propranolol prevented stress-induced oviposition delays, supporting the hypothesis that adrenergic mechanisms are involved in the causation of these delays. It is suggested that adrenaline released in response to stress acts directly to suppress uterine contractions and so to delay oviposition.

# CHAPTER 12: **General Discussion**

The aim of this thesis was to examine the phenomenon of stress-induced oviposition delays in laying hens and to investigate the underlying physiological causation of these delays. The experimental results have been discussed in detail in previous chapters and so the aim of this concluding chapter is to provide a general discussion of these findings and their implications.

In Chapter 3 it was found that social stress (relocation from individual to group cages) caused hens to delay oviposition and this is consistent with the findings of Hughes *et al.* (1986) and Watt (1989). Social stress was then used as the standard environmental stressor in subsequent experiments, where it again induced oviposition delays (see Chapters 4, 6, 7 and 11 and Appendix 3). Other environmental stressors, such as obstructed access to regularly used nest sites, are also reported to delay oviposition (Duncan, 1970; Kite, 1985; Hughes *et al.*, 1986; Cooper & Appleby, 1995). By comparison, oviposition in reptiles and the analogous process in mammals, parturition, are also disrupted by environmental stress (e.g. Haldar & Bade, 1981; Leng *et al.*, 1987; Chan & Solomon, 1989; Guillette *et al.*, 1991; Lawrence *et al.*, 1992).

Several lines of evidence support the assumption that the standard social stress used in this project, which involves both relocation and contact with unfamiliar conspecifics, would be stressful for hens. Relocation of hens from individual cages to a holding crate containing 14 unfamiliar hens is a similar procedure and caused increased plasma corticosterone concentrations (Beuving & Vonder, 1978). Likewise, Gross and Siegel (1985) report that various types of social disruption, including relocation from individual to group cages, increase plasma corticosterone concentration. The release of corticosterone from the adrenal cortex and into the circulation is indicative of stress (Freeman, 1976, 1985). Exposing hens to chronic social disruption (by continuously housing them in groups and regularly changing the composition of these) increases heterophil:lymphocyte ratios and adrenal gland weights and reduces body weight, changes which indicate that social disruption is stressful (Siegel, 1971; Gross *et al.*, 1984; Anthony *et al.*, 1988). Contact between unfamiliar hens is associated with increased levels of aggressive behaviour (Hughes & Black, 1978; Hughes, 1979). Although systematic recordings of hens' behaviour during social stress were not made in the present experiments, the level of aggression at this time was clearly high, relative to that for hens housed in stable groups.

Although social stress was an effective standard stressor for inducing oviposition delays, it was not ideal when designing experiments. Ideally, an independent set of stock hens would be used to make up a stress group for each of the experimental hens (e.g. Section 7.3). However, this was usually impractical as numbers of stock hens were limited. There may be factors related to particular companion hens which influence the severity of the social stress, and so the possibility of group effects must be considered when placing more than one experimental hen in the same stress group. Three experimental designs to reduce or account for possible group effects were used. The first and simplest was to put several experimental hens into each group cage (e.g. Sections 3.3 and 7.2). As these hens had different predicted oviposition times, their periods of stress were due to start and finish at different times. Hence, although the periods when particular hens were exposed to stress may overlap, two hens were not exposed to exactly the same period of stress and so the effects of individual hens on the severity of stress experienced by another should be minimised. The second design was to re-assemble available stock hens into different groups on each day, in such a way that all hens in a group were strangers, and to randomly allocate hens from each treatment to separate groups (e.g. Sections 6.3 and 10.3). This design should thus be balanced for possible effects of individual stock hens. The third design involved pairing experimental hens which had similar predicted oviposition times, and hence whose stress periods were due to start at similar times. One hen from each pair was then randomly allocated to each of the two experimental treatments and both were placed in the same group cage, along with two stock hens. This pairing of hens could then be accounted for in the statistical analysis, thus controlling for possible group effects (see Section 7.4).

A standard stressor that did not involve other hens may be more convenient with respect to experimental design. Although obstructing access to regularly used nest sites appears to potently induce oviposition delays (Duncan, 1970; Kite, 1985; Hughes *et al.*, 1986), it is impractical to accurately measure the daily oviposition times of hens laying in these sites, and so this stressor would not be suitable for experiments of the type reported in this thesis. Relocation to an unfamiliar environment has been used as a model for investigating stress-related disruption of parturition and lactation in mammals (Haldar & Bade, 1981; Leng *et al.*, 1987; Lawrence *et al.*, 1992). Similarly, an appropriate relocation may prove a useful standard stressor for the induction of oviposition delays in hens.

Four mechanisms by which stress might act to delay oviposition were hypothesised in Section 1.5. First, stress may prevent ovarian PG release, so that the

cascade of events which leads to expulsion of the egg is not initiated. Second, stress may inhibit neurohypophysial AVT release so that the AVT-related escalation of uterine contraction frequency does not occur. Third, stress may directly suppress the rise in uterine contraction frequency required for oviposition. Fourth, stress may cause contractions of the utero-vaginal sphincter which prevent the egg from leaving the oviduct. Although the experiments reported in this thesis primarily investigated the second and third mechanisms, all four possibilities are now discussed.

The first hypothesis is that stress causes oviposition delays by preventing ovarian PG release. Treatment with an inhibitor of prostaglandin synthesis such as indomethacin delays oviposition, suggesting that PG release is indeed an essential component of normal oviposition (Hertelendy & Biellier, 1978; Shimada & Asai, 1979). The mechanism controlling the release of ovarian PGs, and therefore the initiation of oviposition, has not yet been described, although it may be modulated by the peak in plasma LH concentration 4-6 h before oviposition (Etches, 1990). At the level of the ovary it is not clear how stress could act to prevent PG release in the present experiments

If stress does cause oviposition delays by preventing ovarian PG release, then plasma PG concentrations are not expected to rise to the levels associated with oviposition during these delays. Although plasma PGFM concentrations were slightly higher during oviposition delays than before expected oviposition time, concentrations at this time were still lower than immediately after oviposition (Chapter 4), and so the possibility that stress suppresses ovarian PG release cannot be discounted. Although the finding that PGF2$\alpha$ injection ends stress-induced oviposition delays is consistent with this hypothesis, the dose administered (0.5 $\mu$g/kg) may have been sufficient to overcome alternative oviposition delaying mechanisms (Chapter 6).

In mammals, environmental stress can act via opioidergic mechanisms to inhibit LH release (e.g. Petraglia *et al.*, 1986; Norman & Smith, 1992). In hens, exogenous opioids can inhibit LHRH (and hence LH) release (Sakuari *et al.*, 1986; Stansfield & Cunningham, 1987; Contijoch *et al.*, 1993). Possibly some environmental stressors could act via opioidergic pathways to inhibit LH release in laying hens and, as LH release is thought to modulate the mechanisms controlling oviposition time (Etches, 1990), could delay oviposition. However, such a mechanism could not have caused the delays observed in the present experiments as the peak of LH release is 4-6 h before oviposition time (Furr *et al.*, 1973; Wilson & Sharp, 1973), and exposure to stress did not start until after this.

The second hypothesis is that stress causes oviposition delays by inhibiting neurohypophysial AVT release. As stress-related disruptions of parturition and lactation in mammals are due to an opioidergic inhibition of oxytocin release (e.g. Haldar & Bade, 1981; Leng *et al.*, 1988; Lawrence *et al.*, 1992), and morphine inhibits osmotically stimulated AVT release in hens (Xu *et al.*, 1991; Dublecz *et al.*, 1992), it was hypothesised that endogenous opioids may inhibit AVT release in hens during stress (see Section 1.7 for a review).

Experiments reported in this thesis investigated plasma AVT concentrations associated with stress-induced oviposition delays, the ability of exogenous AVT to end delays, the effect of opioid receptor antagonism on the delay duration, and the effects of opioid agonists on the oviposition times of undisturbed hens. Plasma AVT concentration was elevated at the time of ovipositions delayed in response to stress but not during the delays (Chapter 4). Injection of exogenous AVT rapidly ended stress-related oviposition delays by expelling the eggs (Chapter 6). Blocking opioid receptors with the non-specific antagonist nalmefene significantly reduced the delay duration (Chapter 7). Stimulating opioid receptors with the selective kappa agonist U50,488 caused oviposition delays, an effect which was prevented by nalmefene and ended by AVT injection (Chapter 8). U50,488 also increased the time from PGF2$\alpha$ injection until premature oviposition was induced and reduced the rise in plasma AVT concentration that is associated with this (Chapter 9). Morphine, a specific mu agonist, may also delay oviposition in some hens (Chapter 8). These findings support the hypothesis that stress-related oviposition delays are due to opioidergic inhibition of neurohypophysial AVT release (see discussions in each chapter).

In mammals, a variety of stressors cause release of endogenous opioids (Rodgers & Randall, 1988) and it is assumed that this also occurs in hens. The finding that exposing hens to social stress reduces their frequency of headflicking in response to alerting stimuli, a putative index of opioid status, is consistent with this assumption (Chapter 10).

In mammals, opioids can inhibit oxytocin release by acting in either the hypothalamus or the pituitary (Bicknell, 1985, 1993). In hens, opioid receptors have not been found on the terminals of AVT neurones (Martin *et al.*, 1992; also see Section 1.6.1), and so opioids presumably act centrally rather than peripherally to inhibit AVT release. Indeed, peripheral inhibition of neurohypophysial hormone release in mammals is via kappa receptors (Bicknell, 1985, 1993) and systematically injected norBNI, a kappa receptor antagonist, did not reduce the duration of stress-induced oviposition delays in hens (Chapter 8). Thus, it is suggested that endogenous opioids (during stress) and exogenous opioids act via central opioid receptors to inhibit AVT

release and so delay oviposition. As endogenous opioids cannot cross the blood brain barrier, it is suggested that the endogenous opioids which inhibit AVT release are released centrally.

In mammals, central opioidergic inhibition of oxytocin release involves mu and kappa but not delta receptors (Bicknell, 1993). In birds, mu, kappa and delta receptors are present in the hypothalamus and so could be associated with AVT neurones (Deviche *et al.*, 1993; also see Section 1.6.2). Although experiments with U50,488 and morphine indicate that delays can be induced by stimulation of kappa and possibly mu opioid receptors, nalmefene is a relatively unselective antagonist and so it is not known which receptor types mediate stress-induced oviposition delays. The disruption of parturition in rats by U50,488, which is thought to involve both central and peripheral kappa receptors, is prevented by 0.5 mg/kg norBNI administered i.v. (Douglas *et al.*, 1993). This suggests that this dose of norBNI is able to antagonise both central and peripheral kappa receptors and so is able to cross the mammalian blood brain barrier. If a similar dose can also antagonise central kappa receptors in the hen, the finding that norBNI does not reduce duration of stress-induced oviposition delays (Chapter 7) would suggest that these are not caused by opioids acting via kappa receptors. However, as it cannot be assumed that norBNI can cross the blood brain barrier in hens, this suggestion would be better supported if it was demonstrated that intracerebroventricular administration of norBNI does not reduce delay duration.

If inhibition of AVT release is the primary mechanism causing oviposition delays during stress, then the stimulus for AVT release, a rise in uterine contraction frequency induced by ovarian PGs, may be expected to occur during the delays. If this stimulus does not occur, then AVT release may not be triggered and hence inhibition of AVT release would not be the primary cause of the delays. Instead, the primary mechanism would be that which prevents the PG induced rise in uterine contraction frequency. In Chapter 4 it was found that plasma PGFM concentrations were slightly higher during stress-induced oviposition delays than before expected oviposition time and this may indicate ovarian PG release. In Chapter 5, SGT recordings did not show a large rise in uterine contraction frequency during oviposition delays. This may be because the stimulus for AVT release did not occur or because it was too short or too small to be detected.

The third hypothesis is that stress-induced oviposition delays are caused by direct adrenergic suppression of uterine contractions. This is supported by reports that exogenous adrenaline causes relaxation of uterine muscle, *in vivo* and *in vitro,* and

induces oviposition delays, all in a propranolol reversible manner, (Verma & Walker, 1974; Crossley *et al.*, 1980; Crossley, 1983); and adrenaline is released in response to stress (Freeman, 1976; Harvey *et al.*, 1984) (see Section 1.7 for a review). The present finding that propranolol prevents the induction of oviposition delays by social stress supports this hypothesis (see Chapter 11 for a full discussion, where it was suggested that propranolol's tranquillising properties may also contribute towards prevention of oviposition delays).

The fourth hypothesis is that stress-related contractions of the utero-vaginal sphincter prevent uterine contractions from expelling the egg. Gilbert & Lake (1963) have described the musculature of this sphincter and it appears suitable for obstructing the egg's passage. If sphincter contractions are the primary cause of stress-related oviposition delays, then ovarian PG release, elevated uterine contraction frequency and AVT release would all be expected to occur as normal during these delays. Although large rises in plasma AVT concentration and uterine contraction frequency were not detected during stress-induced oviposition delays (Chapters 4 and 5), a small rise in plasma PGFM concentration was and this may reflect ovarian PG release (Chapter 4).

Abdominal muscles and body posture are associated with oviposition and facilitate expulsion of the egg (Shimada & Saito, 1989). Presumably changes to these events could contribute towards an oviposition delaying effect of stress. Sturkie *et al.* (1962) describe how hens lay at the expected time when they are treated with curare, a drug which paralyses skeletal muscles but not smooth muscles. The absence of oviposition delays under this procedure, which is presumably stressful, may suggest the involvement of skeletal muscles in the causation of oviposition delays.

The experiments reported in this thesis provide evidence that both adrenergic suppression of uterine contractions and opioidergic inhibition of AVT release are involved in the causation of oviposition delays when hens are exposed to environmental stress. The possibility that other stress-related mechanisms contribute towards causing these delays cannot be excluded. The role of adrenergic and opioidergic mechanisms in the causation of stress-induced oviposition delays is summarised in Figure 12.1.

Presumably the adrenergic and opioidergic mechanisms do not act independently but instead interact to delay oviposition during stress. Possibly the relative importance of these mechanisms varies during exposure to stress, in particular

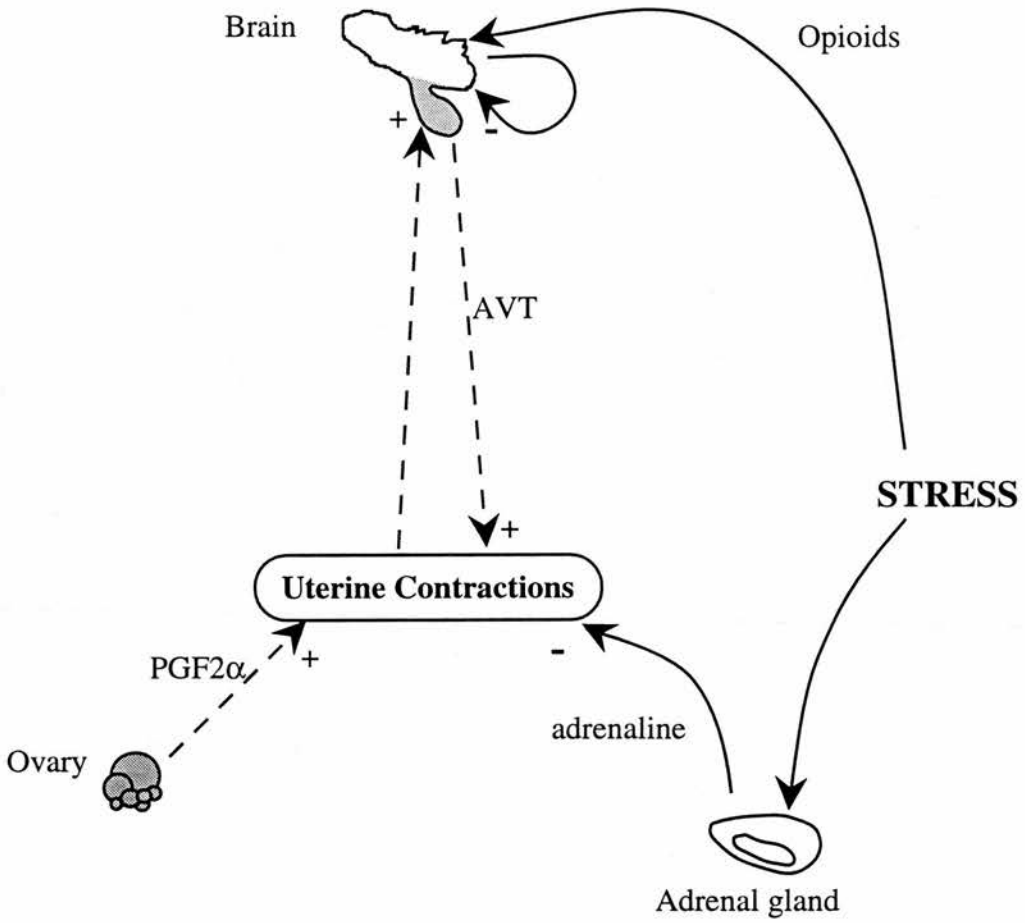



**Figure 12.1** A diagrammatic representation of the mechanisms implicated, by the experiments reported in this thesis, in the causation of stress-induced oviposition delays. Dashed lines represent pathways involved in stimulating uterine contractions, and hence oviposition, in undisturbed hens (see Section 1.4.1 for a review). During stress adrenaline, released from the adrenal glands and into the circulation, directly suppresses uterine contractions and endogenous opioids (probably released and acting centrally) inhibit neurohypophysial AVT release (solid lines).

adrenaline is especially important for mediating immediate responses to stress (Freeman, 1985). As a PG induced rise in uterine contraction frequency is thought to be the stimulus for AVT release (Saito & Shimada, 1989), adrenergic suppression of uterine contractions during stress will presumably suppress AVT release.

Endogenous opioids will also be acting to inhibit AVT release at this time, and so the cascade of events which culminates in expulsion of the egg will not become established. Hens exposed to stress and treated with propranolol do not delay oviposition (Chapter 11), yet AVT release may still be inhibited by endogenous opioids. It is therefore possible that the rise in plasma AVT concentration associated with oviposition will be lower in hens treated with propranolol and exposed to stress than in unstressed hens.

It has been reported that hens continue to lay when they have been either neurohypophysectomised, lesioned in the supraoptic nucleus, or injected with an AVT anti serum at a dose considered sufficient to neutralise all AVT released in association with oviposition (Shirley & Nalbandov, 1956; Ralph, 1960; Opel, 1965; Nakada *et al.*, 1993, 1994). Consequently, it has been suggested that AVT release may not be essential for oviposition (Nakada *et al.*, 1994; also see Section 1.4). If AVT release does not have an important role, then inhibiting its release should not delay oviposition. However, experiments reported in this thesis suggest that this is not the case, because both social stress and exogenous opioids appear to delay oviposition by inhibiting AVT release. The above authors (Shirley & Nalbandov, 1956; Ralph, 1960; Opel, 1965; Nakada *et al.*, 1993, 1994) may, in fact, have missed short oviposition delays because they did not measure oviposition times as accurately as was done here. Thus, although neurohypophysial AVT may not be essential for oviposition to occur, it may be necessary for it to occur at the appropriate time. Sykes (1953a) reported that severing the spinal cord of hens does not prevent ovipositions, although they were usually delayed. If release of neurohypophysial AVT is triggered by a spinal reflex with uterine contractions as the stimulus, a mechanism like that triggering oxytocin release in mammals (Edqvist & Stabenfeldt, 1993), then Sykes' (1953a) finding is consistent with the suggestion above that AVT release is necessary for the appropriate timing of oviposition.

Experiments described in this thesis suggest that endogenous opioids inhibit AVT release during stress and that stimulation of kappa, and possibly mu, receptors can inhibit AVT release in undisturbed hens. Further studies investigating the opioidergic control of AVT release could clarify which receptor types are involved and where they are located. The apparent lack of opioid receptors on the terminals of AVT neurones (Martin *et al.*, 1992, and see Section 1.6.2) suggests that opioids act centrally to inhibit AVT release. In mammals, neurohypophysial hormone release can be triggered by electrically stimulating the neural stalk and this has been used as a tool

for investigating peripheral inhibition of hormone release, both *in vitro* and *in vivo* (e.g. Bicknell *et al.*, 1985; Russell *et al.*, 1993) A similar procedure could be used to investigate the above proposal that opioids do not act peripherally to inhibit AVT release in hens. Opioids which inhibit neurohypophysial hormone release by acting centrally do so by suppressing electrical activity of the magnocellular neurones, and this has been investigated in mammals by directly recording the firing frequency of these neurones (e.g. Pumford *et al.*, 1993). A similar technique could be used to investigate which opioid receptor types can mediate central inhibition of AVT release in hens. In mammals, injection of the peptide CCK causes oxytocin release and this has been used as a tool for investigating opioidergic control of this hormone's release (e.g. Leng *et al.*, 1992). As CCK stimulates AVT release in hens (Marks & Koike, 1993), such an approach could also be useful here.

Ovipositions are delayed when hens are given additional food in the 15 min before expected oviposition time, and this has been interpreted in terms of conflicting feeding and nesting motivations (Freire, 1994; Appleby & Freire, 1995). The present study allows further interpretation of these findings. Firstly, presenting food to the hens may have involved stressful procedures, such as the presence of people, and perhaps it was these disturbances, rather than the food itself, which induced oviposition delays. Secondly, feeding in hens may release endogenous opioids (Savory *et al.*, 1989) and perhaps these cause the delays by inhibiting neurohypophysial AVT release.

As part of the present project, an experiment investigating the effect of food presentation on oviposition time, and the mechanisms underlying any delays that were caused, was attempted. If oviposition delays had been caused by opioidergic mechanisms (related to either feeding behaviour or stress associated with food presentation), then treatment with nalmefene should have reduced their duration. Mature hens of a broiler (meat type) strain were used as these are maintained on a restricted feeding regime and consequently are highly motivated to feed (Savory *et al.*, 1993). However, as these hens did not lay sequences of more than about 6 eggs it was not possible to calculate their expected oviposition times with sufficient accuracy to be able to assess whether or not ovipositions were delayed.

From a commercial perspective, an effect of feeding on oviposition time may be important. Hens are usually fed in the morning (B.O.Hughes & C.J.Savory, personal communication), and this is the time of day when most ovipositions occur (Etches, 1996). If this food presentation delays oviposition then eggs may become dusted, or even white banded in extreme cases, abnormalities which would cause egg

downgrading and possibly compromise the health of embryos (see Section 1.3). Although daily feeding time is reported to influence the distribution of oviposition times (Brake, 1985; Wilson & Keeling, 1991), it is not clear if this was because ovipositions were delayed.

The possibility that oviposition is itself stressful and so stimulates mechanisms which have an inhibitory influence on the expulsion of the egg must be considered. Oviposition is associated with increased heart rate (Mills *et al.*, 1985) and elevated plasma corticosterone concentrations (Beuving & Vonder, 1981; Johnson & Tienhoven, 1981). Both these changes may reflect stress (Freeman, 1976, 1985) and hence are consistent with the possibility that oviposition is stressful. It has been suggested that stress associated with parturition in mammals stimulates adrenergic mechanisms which prolong labour (Bostedt & Rudolf, 1983; Lederman *et al.*, 1985). The finding that propranolol advances oviposition time may be consistent with a similar mechanism occurring in hens (see Chapter 11)

Opioid receptor antagonism reduces inter-birth intervals in rats, suggesting that endogenous opioids may modulate the duration of labour (Leng *et al.*, 1985). In hens, guarding behaviour associated with acute ankle joint pain is not shown during the pre-laying period or at oviposition (Gentle & Corr, 1995), possibly reflecting release of endogenous opioids at this time. If opioids are released they may have an inhibitory effect on AVT release and hence on the progress of oviposition. Indeed, naloxone increases the rise in plasma AVT concentration associated with oviposition (F.Ellendorff, personal communication), suggesting that an endogenous opioidergic inhibition of AVT release is present at this time. However, in the present study nalmefene did not alter oviposition times of unstressed hens (Section 9.6), suggesting that endogenous opioids were not regulating these oviposition times. However, those hens had been laying for about 9 weeks. Possibly sensitivity of the AVT system to opioidergic inhibition declines over successive ovipositions until oviposition is no longer disrupted. The oviposition times of young hens tend to be quite erratic (personal observations), and perhaps this reflects disruption caused by mechanisms related to the stress of oviposition.

The experiments described in Chapter 3 suggest that a threshold of about 3 h oviposition delay defines an upper limit beyond which oviposition of the delayed egg no longer occurs, until the subsequent egg arrives in the uterus several hours later. Before this threshold (i.e. when oviposition delays had not yet reached about 3 h), ovipositions often occurred during stress and if stress ended they occurred shortly

(about 15 min) after. These delays were described as short-term. However, once delays exceeded the 3 h threshold, ovipositions were unlikely to occur during stress or even if stress ended. Instead the eggs were laid much later, 7-15 h after expected oviposition time, and these delays were described as long-term. Thus, the threshold describes the time after expected oviposition time beyond which oviposition no longer occurs until much later. Four mechanisms to explain this threshold were hypothesised in Chapter 3, and these are now considered in the light of subsequent experiments.

First, release of ovarian PGs, the initial event in the oviposition process, may be restricted to a short time period which has an upper limit defined by the threshold. As the mechanisms controlling ovarian PG release have not been described, this hypothesis has not been tested.

Second, neurohypophysial AVT and/or ovarian PGs may be released during stress-induced delays and the threshold is the time beyond which stores of these hormone(s) are so depleted that oviposition can no longer occur. In Chapter 4 there was no evidence to suggest that AVT is released during oviposition delays. Indeed, the findings in subsequent chapters suggest that inhibition of AVT release is one of the main causes of oviposition delays. Thus, it is unlikely that the threshold reflects depletion of neurohypophysial AVT stores. Plasma PGFM concentrations were slightly higher during stress-induced oviposition delays than before expected oviposition time, possibly reflecting release of ovarian PGs (Chapter 4). Therefore, the possibility that the threshold reflects a depletion of ovarian PG stores (and presumably their precursors) cannot be discounted.

Third, the oxytocin (and presumably AVT) degrading enzyme activity in plasma and uterine tissues is lowest at oviposition time but rises within a few hours (Gilbert & Lake, 1964; Brzezinska *et al.*, 1967; Brezezinska-Slebodzinska *et al.*, 1979). Hence, the threshold may reflect the time beyond which any AVT released is degraded so rapidly that it does not stimulate uterine contractions. Conceivably, a similar mechanism could also apply to PGs. Injections of either AVT or PGF2$\alpha$ ended long-term oviposition delays either very slowly or not at all (Section 6.4). By contrast, similar injections rapidly end short-term delays (Section 6.3). This is consistent with the hypothesis that reductions in the half-lives of these hormones cause the threshold.

Fourth, changes in the number and binding characteristics of uterine receptors for AVT and/or PGs may alter, reducing the ability of these hormones to induce uterine contractions after the threshold. Such changes have been described for the AVT receptor and may be under the control of ovarian hormones (Takahashi *et al.*,

1992, 1994). This hypothesis is also consistent with the finding that neither AVT nor PGF2α injections readily end long-term oviposition delays (Section 6.4).

Thus, a single mechanism causing the threshold has not been identified and all four discussed above could contribute.

In an experiment examining the amenability of oviposition in hens to operant conditioning, Leuscher and Hurnik (1988) were unable to train hens to lay in the late afternoon (rather than in the morning as normal) in order to get a food reward. As hens do not normally feed during the pre-laying period (Savory, 1977; Shimada *et al.*, 1987b), it was suggested that there may be "associative constraints" between oviposition as a behaviour and food as a reward. The identification of the 3 h threshold (Chapter 3) suggests another interpretation of their experiment. Once oviposition has been delayed beyond this threshold hens no longer lay eggs until the subsequent egg arrives in the uterus. Thus, the hens in the experiment of Leuscher and Hurnik (1988) may have been unable to lay at the required time as this was after the threshold. Possibly oviposition would be more amenable to operant conditioning if rewarded changes in oviposition time were within the period defined by the 3 h threshold.

In conclusion, there are several points raised by the present study which merit further investigation. First, it would be of interest to investigate the underlying cause of the 3 h threshold, which affects the duration of oviposition delay, and hence the type of eggshell abnormality. Second, further investigation of opioidergic modulation of neurohypophysial AVT release could concentrate on identifying the sites of action of the opioid receptor types. Third, further studies are required to validate the use of headflick frequency as a behavioural indicator of hens' opioid status. Fourth, the regulation of ovarian PG release at normal oviposition is still poorly understood (Shimada & Saito, 1989). Investigation of this and its possible disruption by stress is needed. Fifth, Some consideration of the possible contribution of stress-related adrenaline release on contractions of the utero-vaginal sphincter is required. Finally, investigations with electrodes are required to study uterine contraction patterns in relation to normal and delayed ovipositions, and associated pharmacological manipulations.

# Summary of Findings

1. Exposure to social stress (relocation from individual to group cages for periods of up to 6 h) at expected oviposition time caused many hens to delay oviposition. These delays often ended during stress, and if exposure to stress ended oviposition typically ended about 15 min after this. Ovipositions delayed beyond a threshold of about 3 h were unlikely to occur until they had been delayed for 7-15 h, by which time the subsequent egg had arrived in the uterus. Delays ending before the threshold were described as short-term while those ending later were described as long-term.

2. Short-term oviposition delays often resulted in the delayed eggs acquiring a superficial layer of additional calcification, an abnormality described as "dusting". When oviposition was delayed long-term the delayed egg became "white-banded" and the subsequent egg became "slab-sided".

3. Plasma concentrations of both prostaglandin-FM and AVT were elevated at the times of both normal ovipositions and ovipositions occurring after short-term stress-induced delays. Prostaglandin-FM, but not AVT, concentrations were slightly elevated during oviposition delays.

4. Strain gauge transducers were used to record uterine contractions in association with normal oviposition, oviposition induced prematurely by exogenous PGF2α, and one oviposition delay induced by stress (relocation). Contraction frequency rose two-fold (from about 10-12 per min to about 20-25 per min) at each of these ovipositions. There was no evidence of increased contraction frequency during the oviposition delay.

5. Social stress did not affect the ability of exogenous PGF2α (0.5 μg/kg, i.v.) to induce oviposition 2 h prematurely.

6. I.v. injection of either AVT (0.1 μg/kg) or PGF2α (0.5 μg/kg), but not of saline, rapidly ended short-term stress-induced oviposition delays.

7. I.v. injection of either AVT (0.1 μg/kg) or PGF2α (0.5 μg/kg) ended only some (2/4 and 1/4, respectively) long-term stress-induced oviposition delays, and then only relatively slowly.

8. The relatively unselective opioid receptor antagonist nalmefene (0.5 mg/kg, i.v.) significantly reduced the duration of stress-induced oviposition delays.

9. The preferential kappa and mu receptor antagonist MR2266 (2 mg/kg, i.v.) reduced the duration of stress-induced oviposition delays, but this effect was not significant.

10. The selective kappa opioid receptor antagonist norBNI (0.5 mg/kg, i.v.) did not significantly affect the duration of stress-induced oviposition delays.

11. Treatment with the selective mu receptor agonist morphine (6 mg/kg, i.v.) induced some hens' ovipositions within 8-13 min and these were 28-101 min premature. For some other hens this treatment may have delayed oviposition.

12. Treatment with the selective kappa receptor agonist U50,488 (4 mg/kg, i.v.) caused oviposition delays with a mean duration of 26.4 min. The oviposition delaying effect of U50,488 was prevented by co-treatment with nalmefene (0.5 mg/kg, i.v) and ended by exogenous AVT (0.1 μg/kg, i.v.).

13. Treatment with U50,488 (4 mg/kg, i.v.) delayed the premature induction of oviposition by exogenous PGF2α (0.5 μg/kg, i.v.) and reduced the rise in plasma AVT concentration associated with this. These effects were not apparent in hens co-treated with nalmefene (0.5 mg/kg, i.v.).

14. In one experiment, social stress significantly reduced hens' frequency of headflicking in response to alerting stimuli. In a second experiment, frequency of headflicking was significantly increased by nalmefene (0.5 mg/kg, i.m.) but was not significantly affected by social stress. The i.m. injection procedure (with saline) did not significantly affect headflick frequency.

15. The β adrenergic receptor antagonist propranolol (10 mg/kg, i.m.) prevented stress-induced oviposition delays. This treatment also caused ovipositions of unstressed hens to occur on average 25 min earlier than expected.

16. In addition to causing oviposition delays, social stress can induce relatively short (< 1 h) ovulation delays (see Appendix 3).

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# Appendix 1: **Validation of Expected and Predicted Oviposition Times**

In many of the experiments reported in this thesis it was important that hens could be treated a specific time interval before they would normally lay. To enable this, the time when oviposition would normally occur was predicted using records of previous days oviposition times. The "predicted oviposition time" is therefore an estimate of when oviposition would occur (if there was no experimental treatment) and was a means of determining when experimental procedures should be carried out. The "expected oviposition time" is a more accurate estimate of when oviposition would have occurred (had there been no experimental treatment) and is calculated using the recorded oviposition times on the days before and after treatment. In experiments expected oviposition time was used in the calculation of oviposition delays.

There were two aims in the present study: first, to assess how accurately expected oviposition time represents actual (observed) oviposition time in the absence of any experimental treatments; second, to assess how accurately predicted oviposition times reflect expected oviposition times.

## A1.1 Materials and Methods

A stock of hens (n = 37) aged 28 weeks were housed and their oviposition times recorded as described in Chapter 2. After the third day of recording, the oviposition time of the egg due on day 4 could be predicted (predicted oviposition time) for 27 of the hens. Observed oviposition times of these hens were then recorded for a further 3 days (days 4, 5 and 6) and the occurrence of oviposition on day 7 recorded (to ensure that day 4 was not within three days of the end of an egg sequence). For each of the 27 hens, the expected oviposition time for day 4 was calculated twice; once using the observed oviposition times on days 3 and 5 (first day after day 4) and once using the observed oviposition times on days 3 and 6 (second day after day 4), as described in Section 2.3. In this project, expected oviposition time for a day of treatment was only calculated using the second day after treatment when oviposition time on the first day after treatment was not known, e.g. as a result of an internal ovulation.

To assess how accurately expected oviposition times reflected observed oviposition times, the expected oviposition times were deducted from the observed oviposition times (this was done twice; once for each method of calculating expected oviposition time). Thus, a value of zero indicates that expected oviposition time

equalled observed oviposition time, negative values indicate that expected oviposition time was later than observed oviposition time and positive values indicate that expected oviposition time was earlier than observed oviposition time. Note that these values are equivalent to oviposition delays.

To assess how accurately predicted oviposition time reflected expected oviposition time, the predicted oviposition times were deducted from the expected oviposition times. Thus a value of zero indicates that predicted oviposition time equalled expected oviposition time, negative values indicate that predicted oviposition time was later than expected oviposition time and positive values indicate that predicted oviposition time was earlier than expected oviposition time. As most expected oviposition times for the experiments in this project were calculated using the oviposition time on the first day after treatment, the accuracy of predicted oviposition times was assessed here using this calculation (i.e. using day 5).

## A1.2 Results and Discussion

One hen laid a soft shelled egg very early and clearly prematurely on day 5 and so its expected oviposition could only be calculated using day 6. For another hen, the oviposition time on day 4 was not recorded and so this individual could not be included in the analysis.

Figure A1.1 shows the distribution of differences between observed oviposition times and expected oviposition times (calculated by both methods) for day 4. Both distributions were normally distributed and so confidence intervals could be calculated. The 95% confidence interval of expected oviposition times calculated using day 5 (the first day after day 4) was within -29 and +28 min (mean = 0 min) of observed oviposition time. The 95% confidence interval of expected oviposition times calculated using day 6 (the second day after day 4) was within -32 and +27 min (mean = -2 min) of observed oviposition time.

This study demonstrates that calculations of expected oviposition time used in this project give an accurate approximation of observed oviposition time in the absence of experimental treatments. Thus, in experiments to investigate the effects of treatments on oviposition time, expected oviposition times can be used to estimate when ovipositions would have occurred in the absence of the treatments.

Figure A1.2 shows the distribution of differences between expected oviposition times (calculated using day 5) and predicted oviposition times. The data were normally distributed and so confidence intervals could be calculated. The 95%

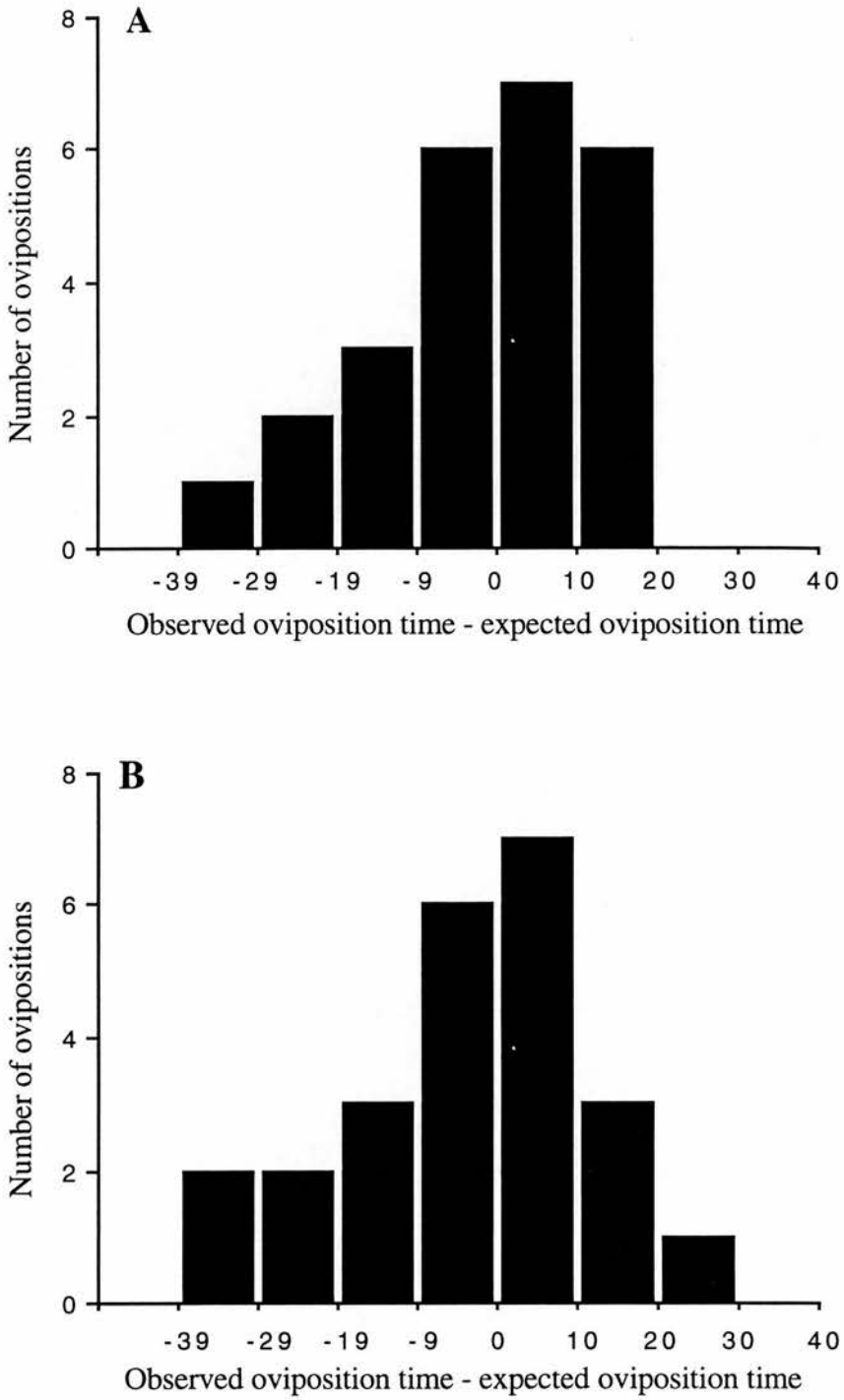


**Figure A1.1** Distributions of differences between observed oviposition time and expected oviposition time, calculated either using day 5 (A; first day after day 4) or day 6 (B; second day after day 4), and observed oviposition time. Data are grouped in classes of 10 min.

confidence interval of predicted oviposition times calculated using day 5 was within -45 and +26 min (mean = -5 min) of expected oviposition time. Thus, expected oviposition time can be predicted in advance with a level of accuracy sufficient for the experimental protocols described in this thesis. As a result of the variation in predicted oviposition time accuracy, it was not possible to treat different birds at exactly the same time relative to their expected oviposition times, and consequently in all experiments there is a range of times from the treatments until the expected oviposition times.

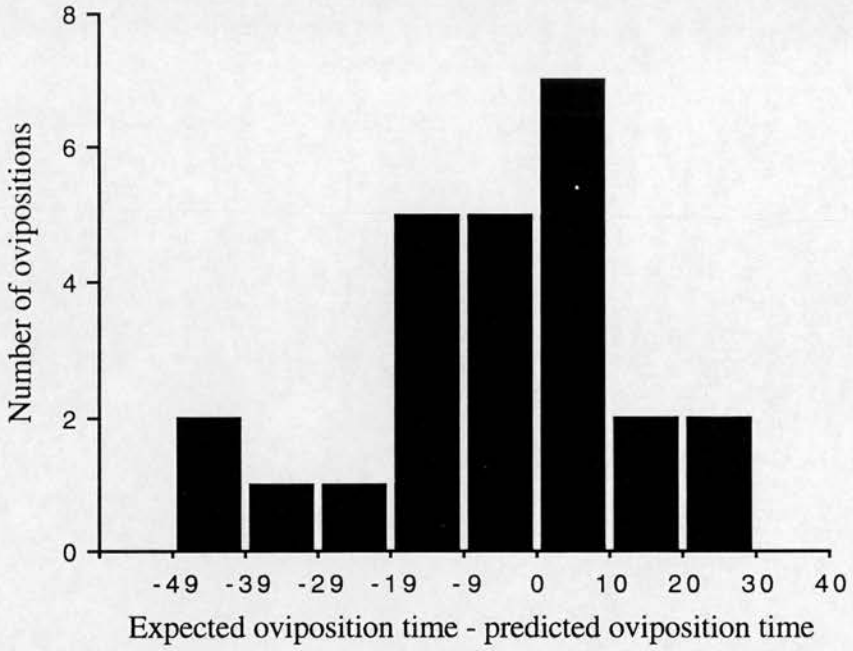


**Figure A1.2** Distributions of differences between expected oviposition time and predicted oviposition time. Data are grouped in classes of 10 min.

The hens used in this study were of a similar age to those used in most of the experiments described in the thesis. The proportion of stock birds selected in the present study is also similar to that in most other experiments. However, it should be noted that the accuracy of expected oviposition time calculations and of predicted oviposition times may differ from those recorded here in some experiments, e.g. in older birds where the lag is longer and egg sequences shorter (Lillpers & Willhelmson, 1993a).

# Appendix 2: **Validation of the Radioimmunoassay used to Measure AVT in Avian Plasma**

## A2.1 Introduction

As there was no commercially available assay for the measurement of AVT, AVP radioimmunoassay kits were used to measure plasma AVT concentrations in this thesis (Chapters 4 and 9). AVT and mesotocin are the avian equivalents of mammalian AVP and oxytocin, respectively - all are nonapeptides and are comprised of a ring and tail component (Acher, 1993). The human AVP assay kit used (RIK-8103; Peninsula Laboratories, Inc.) was chosen, from a number of oxytocin and AVP assay kits available, because the peptide antiserum is reported to have 100% cross-reactivity with AVT (antiserum specifications supplied with kit). This appendix describes the protocol of this radioimmunoassay and the steps taken to validate its use for measuring AVT in avian plasma.

Radioimmunoassays are based on the competition between hormone which has been labelled with a radioisotope and unlabelled hormone (either in a standard or extracted from a plasma sample) for a limited number of binding sites on specific antibodies (see Chard (1990) for a review of assay principles). The relationship between the amount of labelled hormone binding to the antibodies and the amount of unlabelled hormone is described by a sigmoidal standard curve. The amount of labelled hormone which binds to the antibodies when in competition with an unknown amount of unlabelled hormone (e.g. hormone extracted from plasma) is compared with the standard curve to determine the amount of unlabelled hormone. In the assay described here, unlabelled AVT (standard or from plasma) competes with a fixed amount of $^{125}I$ labelled AVP.

## A2.2 Assay Procedure

Assays were conducted according to the instructions supplied with the kits, with the exception that a modified extraction procedure was used. The modifications to the extraction procedure were required because plasma from laying hens contains larger amounts of lipoprotein than human plasma (for which the original extraction procedure was designed).

*Extraction of Peptide from Plasma*

Plasma (0.5 ml) was mixed with 1 ml buffer A (0.1% Trifloro acetic acid (anistar grade; Merck) in distilled water), then centrifuged for 20 min at 750 **g** to pellet precipitated proteins (the original extraction procedure used 0.5 ml buffer A). With a Pasteur pipette the supernatant was loaded onto a Sep pac vac C-18 column (vac-3cc, Waters Associates, Millipore, UK), which had been prepared by washing once with 1 ml buffer B (60% acetonitrile (Rathburn Chemicals) in buffer A) then three times with buffer A, and then slowly drawn through. The column was then washed twice with 3 ml buffer A and then, to elute the peptide, slowly with 3 ml buffer B. This final eluant was collected and evaporated to dryness overnight at 35°C in a vacuum oven (pressure = 780 mm Hg). The peptide was then reconstituted with assay buffer. During the extraction procedure, solutions were slowly drawn through the C-18 columns, at 2 ml/min, using water pressure to create a vacuum.

*Summary of Assay Protocol*

[ARG$^8$]-vasotocin acetate (Sigma) was used as the standard AVT in the preparation of the standard curves and in the steps taken to validate the assay. Its structure is identical to that of avian AVT (Acher, 1993).

All standards were replicated and then they and the extracted samples were assayed in duplicate. Samples of standard peptide and peptide extracted from plasma were incubated with rabbit anti-AVP serum overnight at 4°C. $^{125}$I-AVP was then added followed by a second overnight incubation at 4°C. Goat anti-rabbit IgG serum and normal rabbit serum were then added, followed by 1.5 h incubation at room temperature. After centrifugation at 1700 **g** for 20 min, the supernatant was removed from each tube by aspiration and the $I^{125}$ content of the pellets counted using a gamma counter (1277 Gammamaster, Wallac). A radioimmunoassay curve fitting programme (Assay Zap) was used to analyse the assay results.

## A2.3 Assay Specifications and Validation

Several steps were taken to demonstrate that the radioimmunoassay kits for measuring AVP in mammalian plasma could be used for measuring AVT in avian plasma. Firstly, standard curves of AVP and AVT were prepared. Parallelism

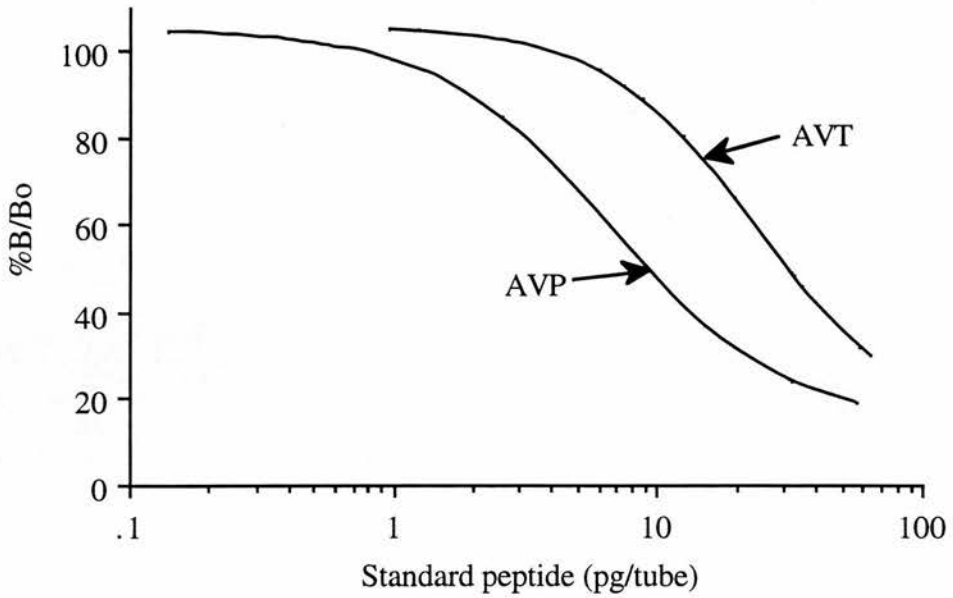


**Figure A2.1** AVT (prepared in phosphate buffered saline) and AVP standard curves.

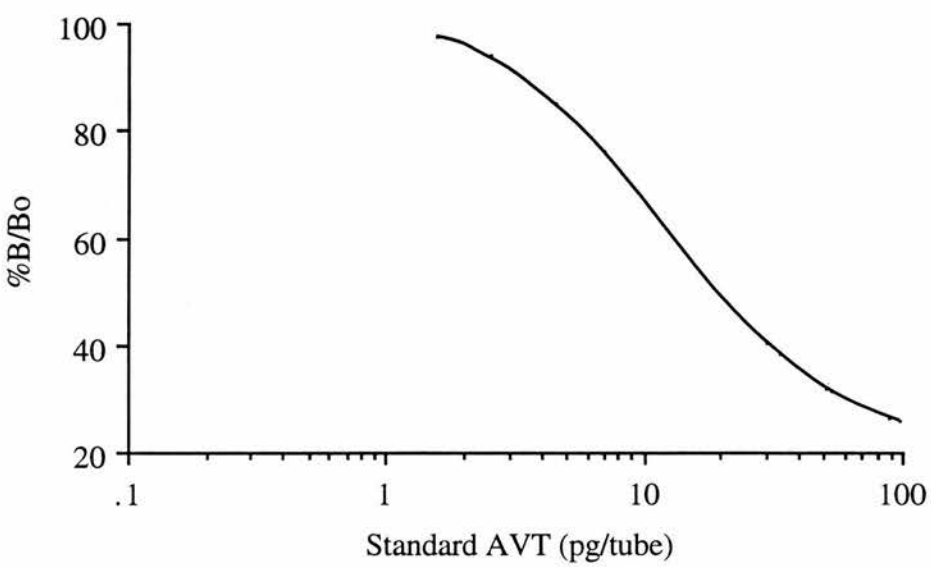


**Figure A2.2** Standard curve for AVT, as used for measuring AVT from plasma.

between these indicates that unlabelled AVT can compete with labelled AVP for specific antibody binding sites. It was also confirmed that the expected plasma AVT concentrations would fall within the range where the standard curve has an almost linear slope. Secondly, various known amounts of AVT were added to stock plasma. These increases in plasma AVT concentration were accurately measured by the assay, indicating that there is a linear relationship between measured and actual plasma AVT concentration. This procedure was also used to calculate the efficiency of the extraction procedure. Finally, the plasma AVT concentrations measured in association with oviposition were compared with published reports.

*AVT and AVP Standard Curves*

In a preliminary assay, standard curves for AVP and AVT were prepared by two-fold serial dilutions of standard AVP and standard AVT. As there was insufficient assay buffer supplied with the kit for preparing two standard curves, the serial dilutions of AVT were made using phosphate buffered saline (for subsequent assays, including the rest of the validation procedures, the AVT standard curve was prepared with assay buffer). The parallelism between the AVP and AVT standard curves (Figure A2.1) indicates that AVT can compete with $^{125}$I-AVP for binding to the specific antibodies. The slight displacement of the AVT standard curve to the right of the AVP standard curve may indicate that AVT binds less competitively with the antibodies than does AVP. In subsequent assays, the lowest AVT standard was replaced with a higher one in order to improve the range of AVT determination. Such an AVT standard curve is shown in Figure A2.2. For this standard curve, ED80 = 3.1 pg/tube, ED50 = 12.6 pg/tube, ED20 = 57.0 pg/tube.

*Recovery of AVT Added to Plasma*

To confirm that the assay could accurately measure the addition of various amounts of standard AVT added to plasma, and to assess the efficiency of the extraction procedure, either 0, 20, 40 or 60 pg/ml (n = 4, 2, 2, and 2, respectively) of standard AVT was added to aliquots of pooled stock plasma. To calculate the amount of added AVT which was recovered (measured) by the assay, the mean AVT concentration measured for plasma samples without added AVT (this was 21.1 pg/ml) was deducted from the mean AVT concentrations measured for plasma samples with AVT added at 20, 40 or 60 pg/ml. Mean plasma AVT concentration recovered was directly proportional to the amount of added plasma, i.e. the percentage recovery was similar for each amount of added AVT (Table A2.1). Thus, the assay can accurately measure the addition of known amounts of AVT to avian plasma. Mean recovery of

added standard AVT, an estimate of the efficiency of the extraction procedure, was 86%. In experiments, measurements of AVT from plasma were multiplied by 1.16 (i.e. 100/86) to account for the extraction procedure.

| AVT added to stock plasma (pg/ml) | 20 | 40 | 60 |
|---|---|---|---|
| Mean AVT recovered (pg/tube x2) | 17.7 | 31.4 | 54.7 |
| Recovery of added AVT (%) | 88.4 | 78.4 | 91.2 |

**Table A2.1** Recovery of standard AVT added to stock plasma. As AVT was measured in 0.5 ml of plasma, AVT recovered is expressed as pg/tube x 2.

*Assay Variation*

It was intended that the variation between the 4 replicate measurements of AVT from stock plasma spiked with no added AVT (0 pg/ml) would be used as an estimate of intra-assay variation. However the variation between these replicates was clearly higher than the variation between replicates of other samples (including those containing added AVT). Therefore, to get a more representative estimate of intra-assay variation the replicate measurements of AVT in plasmas to which AVT had been added at either 20, 40 or 60 pg/ml were also used. The difference between each replicate and the mean for that set of replicates was calculated as a percentage of the mean. Thus, 10 estimates of variation were calculated and the mean of these, 17.9%, was taken as an estimate of the intra-assay variation. If the plasmas with no added AVT are excluded, the estimate of intra-assay variation is 8.6%. It is not clear why there was high variability in the plasma with no added AVT.

So that an estimate of inter-assay variation could be made, two samples of stock plasma with no AVT added (0 pg/ml) were included in a subsequent assay. The mean of the 6 replicate measurements of AVT in this plasma (including the 4 from the earlier assay) was calculated. The difference between each replicate and the mean was calculated as a percentage of the mean. The mean of these 6 estimates of variation, 24.4% was taken as an estimate of inter-assay variation. As there was much variation between the replicates in the earlier assay (see above), the estimate of inter-assay variation is very high. Within each of the experiments described in this thesis, all plasma samples were included in the same AVT assay.

*Comparison Between Published and Measured AVT Concentrations*

Plasma AVT concentration is reported to rise to about 4 or 5 times basal level at the time of oviposition (Arad & Skadhauge, 1984; Tanaka *et al.*, 1984; Rice *et al.*, 1985; Shimada *et al.*, 1986; Koike *et al.*, 1988). Using the assay described here, a five-fold rise in plasma AVT concentration was measured in association with oviposition (Chapter 5). This supports the conclusion that the present assay does indeed measure AVT in avian plasma.

# APPENDIX 3: **Are Stress-Induced Oviposition Delays Associated With Delay of the Subsequent Egg's Ovulation?**

## A3.1 Introduction

This thesis has confirmed that social stress can delay oviposition in laying hens. Ovipositions of undisturbed hens are usually followed by ovulation of the subsequent egg 15-45 min later (Bahr & Johnson, 1991). Although ovulation is caused by the pre-ovulatory surge in plasma LH concentration which occurs 4-6 h earlier, the mechanisms underlying this association are not fully understood (see Etches (1996) for a recent review). In the pre-ovulatory period, proteolytic enzymes and collegenase weaken the stigma region of the largest pre-ovulatory follicle. Ovulation then occurs when the stigma ruptures releasing the ovum. With respect to eggshell quality and number of eggs produced it is of interest to know if ovulation of the subsequent egg is delayed in association with oviposition delays induced by social stress. Thus the aim of the following study was to investigate the effect of social stress on the timing of ovulation.

In both experiments reported here hens were either exposed to social stress, to induce oviposition delay, or left undisturbed (control), to allow oviposition to occur normally, and then the state of ovulation was assessed by post-mortem examination. In Experiment 1, all hens were killed at observed oviposition time to compare effects of stress and control treatments on state of ovulation at this time. In Experiment 2, all hens were killed 35 min after predicted oviposition time to compare effects of stress and control treatments on state of ovulation at this time. From Experiment 2 it was concluded that social stress can delay both ovulation and oviposition. In Experiment 1 there was no evidence of oviposition delays exceeding 1 h and so it was concluded that the ovulation delays caused by social stress may be relatively short.

## A3.2 Experiment 1: Effect of Stress on the State of Ovulation at Oviposition Time

The aim of this experiment was to compare the states of ovulation of the subsequent egg at the times of delayed oviposition and normal oviposition.

### A3.2.1 Materials and Methods

From 39 stock hens aged 40 weeks, 23 whose next oviposition times could be predicted were selected and allocated randomly to either control (n = 11) or stress (n = 12) treatments. Control hens were left undisturbed in their original cages, while hens due to be stressed were placed in a plastic holding pen (0.90 m long x 0.65 m wide x 0.58 m high) in an unfamiliar room 30 min before their predicted oviposition times. The number of hens in this tub was maintained at eight by using unselected stock birds. All hens in each treatment were killed by injection of sodium pentobarbitone (sagatal, Rhone Merieux) < 2 min after their observed oviposition or 4 h after predicted time if they had not laid before this. Within 3 min of death the oviduct was exposed and the position of the next ovum was classified as either in the ovary (not ovulated), body cavity (ovulated but not yet taken up by the infundibulum), infundibulum, magnum, or isthmus.

As expected oviposition times could not be calculated from subsequent oviposition times (because the hens were dead), oviposition delays were calculated by deducting the predicted oviposition time from the observed oviposition time.

### A3.2.2 Results

Of the 12 hens exposed to the stress treatment, 5 did not lay and were killed 4 h after predicted oviposition time. All 11 control hens laid within 1 h of their predicted times. When they were killed, only 1 of the control hens had not ovulated while 9 of those exposed to stress had not. This difference between the treatments was significant (Fishers exact test, $P<0.01$), indicating that hens exposed to stress were more likely to have ovulated before they laid than undisturbed birds.

As the maximum delay of 4 h was reached by 5 hens exposed to stress, the durations of oviposition delays following each treatment were compared using rank statistics. The oviposition delays of hens exposed to stress were significantly longer than those of control hens (Mann-Whitney, w=83, $P<0.01$).

Figure A3.1 shows the relationship between the duration of oviposition delay and the position of the egg in the oviduct and indicates that ova ovulated in association with longer oviposition delays were in a more advanced position in the oviduct than those associated with short oviposition delays. It is possible that some ova found in the body cavity may have been dislodged from the infundibulum during the post-mortem examination. The positions in the oviduct of all the ova were consistent with published reports on normal ovulation. The developmental stages of ova were

appropriate to their positions in the oviduct, with those in the magnum carrying albumen and those in the isthmus carrying shell membranes as well.

These results suggest that ovulation is not delayed in association with stress-induced oviposition delays. However, examining the positions of ovum in the oviduct several hours after ovulation may not be a very accurate method for determining ovulation time and so it is possible that stress may have induced short ovulation delays (i.e. not longer than 1 h).

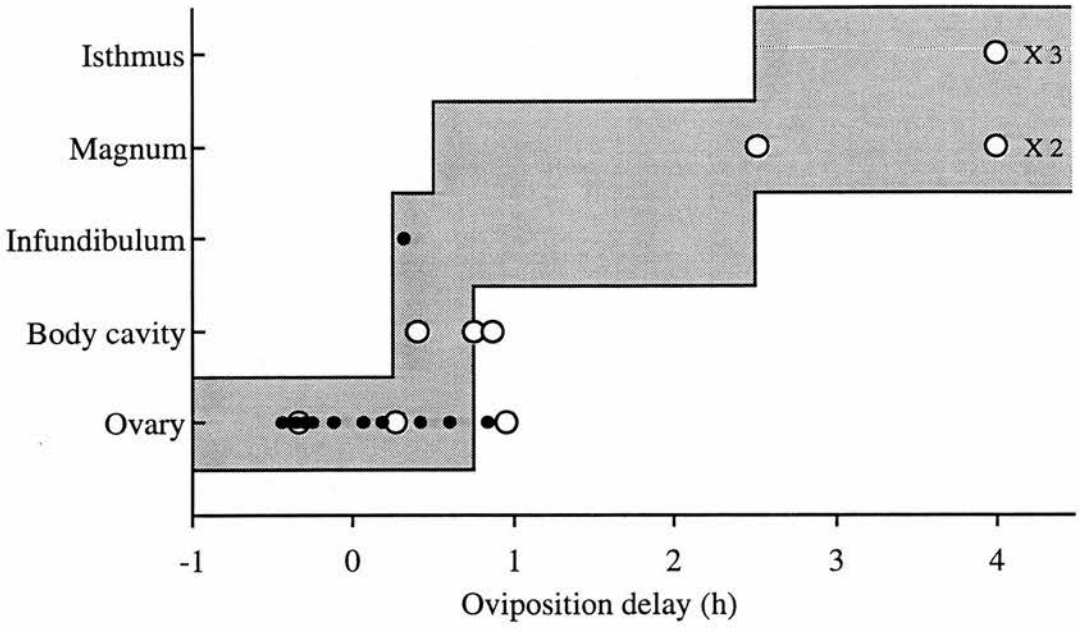


**Figure A3.1** The relationship between the duration of oviposition delay and the position of the next ovum in the oviduct for hens either exposed to stress (large open circles) or not disturbed (small solid circles). The five birds with delays of 4 h had not laid when they were killed. The shaded regions represent the range of expected positions of the ova at different times after expected oviposition time (Bahr & Johnson, 1991).

## A3.3 Experiment 2: Effect of Stress on the Stage of Ovulation 35 Minutes After Predicted Oviposition Time

In Experiment 1, ovulation (of the subsequent egg) preceded oviposition more frequently in hens exposed to social stress than in control hens. Hens exposed to stress laid after longer oviposition delays than the control hens and the positions of the recently ovulated ova in the oviduct and their stages of development were compatible with normal passage in the oviduct, suggesting that ovulation was not delayed in association with the stress-induced oviposition delays. Experiment 2 tested this conclusion by investigating the effects of stress and control treatments on the stage of ovulation 35 min after predicted oviposition time. This should be a more sensitive way to detect ovulation delays.

### A3.3.1 Materials and Methods

From 69 stock hens, aged 34 weeks, 55 whose next oviposition times could be predicted were selected and allocated randomly to either control (n = 27) or stress (n = 28) treatments. Control hens were left undisturbed in their original cages while hens due to be stressed were placed in a plastic tub (as in Experiment 1) 40 min before their predicted oviposition times. The number of hens in the tub was maintained at 6-11 using unselected stock birds. All hens were killed by injection of sagatal 35 min after their predicted oviposition time and the status of the subsequent ova were classified as ovulated or not ovulated. This time of death was chosen as ovulation normally occurs 15-45 min after oviposition (Bahr & Johnson, 1991), and it could therefore be expected that most of the control birds would have ovulated before they were killed.

Oviposition times were recorded and oviposition delays were calculated by deducting the predicted oviposition time from the observed oviposition time, as in Experiment 1.

### A3.3.2 Results

On the day of treatment, two hens, one from each treatment, did not carry hard-shelled eggs and were excluded from the experiment (so, n = 26 for control treatment and n = 27 for stress treatment).

For stress and control treatments, Table A3.1 shows the numbers of hens which ovulated or did not ovulate before 35 min after predicted oviposition time.

Compared with those receiving the control treatment, a significantly greater proportion of the birds exposed to stress had not ovulated ($X^2$=16.2, 1 df, $P$<0.001). Thus, the stress treatment delayed the time of ovulation.

For stress and control treatments, Table A3.2 shows the numbers of hens which laid or did not lay before 35 min after predicted oviposition time. Compared with those receiving the control treatment, a significantly greater proportion of the hens exposed to stress had not laid ($X^2$=45.3, 1 df, $P$<0.001). Only one hen allocated to the stress treatment laid and this oviposition occurred before the stress treatment started (i.e. oviposition was more than 40 min before predicted oviposition time) and so no hens laid during their exposure to stress. This hen was one of the 4 which ovulated before 35 min after predicted oviposition time. One of the hens receiving the control treatment also laid more than 40 min before predicted oviposition time. The median oviposition delay for hens in the control treatment was 0 min, so these birds laid at around the predicted time. Thus, compared with the control treatment, the stress treatment delayed the time of oviposition.

| Treatment | Ovulated | Not ovulated |
|---|---|---|
| Control | 18 | 8 |
| Stress | 4 | 23 |

**Table A3.1** Numbers of hens which, at 35 min after predicted oviposition time, had either ovulated or not ovulated.

| Treatment | Laid | Not laid |
|---|---|---|
| Control | 25 | 1 |
| Stress | 1 | 26 |

**Table A3.2** Number of hens which, at 35 min after predicted oviposition time, had either laid or not laid.

## A3.4 Discussion

In Experiment 1, hens exposed to stress were significantly more likely to have ovulated before laying the egg currently in the uterus and also had significantly longer oviposition delays than undisturbed hens. The positions of the recently ovulated ova at the times of ovipositions delayed for various durations suggest that these ovulations occurred at their normal times, and hence that the stress-induced oviposition delays were not accompanied by ovulation delays. To use the position of the ova in the oviduct as an indicator of ovulation time relies on the assumption that the rate of the ova's passage through the oviduct is not altered by the stress. The stages of development of the ova recovered from the magnum and the isthmus were similar to those reported in the literature (Roberts & Brackpool, 1994) and this is consistent with the assumption that passage through the oviduct was not altered by the stress. However, examining the position of ovum in the oviduct may not be a very sensitive method for estimating ovulation time and so the possibility that short (i.e. less than 1 h) ovulation delays are associated with exposure to stress cannot be excluded.

Experiment 2 investigated the stage of ovulation 35 min after predicted oviposition time as this should give a more sensitive (than Experiment 1) indication of whether ovulation is delayed by social stress. Relative to the control treatment, social stress greatly reduced the proportion of hens which had ovulated and the proportion of hens which had laid before 35 min after predicted oviposition time, indicating that both ovulations and ovipositions were delayed. From Experiment 2 it is not possible to estimate the duration of the ovulation delays that were caused. However, as there was no evidence of long ovulation delays in Experiment 1, it seems likely that the ovulation delays caused by social stress are relatively short (< 1 h).

The timing of ovipositions of white-banded eggs also suggests that ovulation delays associated with social stress are likely to be short. When oviposition is delayed beyond a threshold of about 3 h, the delayed egg is not laid for several more hours, during which time the next egg ovulates and eventually arrives in the uterus (Chapter 3). Where these eggs touch, the delayed egg acquires a white-band and the recently ovulated egg acquires a slab-side. Ova ovulating at the normal time do not reach the uterus until 4-5 h after the previous egg's normal oviposition time (Bahr & Johnson, 1991), yet white-banded eggs may be laid after oviposition delays of as short as 7 h duration (Chapter 3). Shell material is deposited slowly during the first 3-4 h an egg spends in the uterus (Talbot & Tyler, 1974; Nys, 1986), and presumably it takes a

period of contact between the delayed and recently ovulated eggs for them to acquire their white-bands and slab-sides. Thus, allowing time for the subsequent egg to reach the uterus and time for the white-band and slab-side shell defects to form, the timing of ovipositions of eggs with white-bands also suggests that ovulation delays associated with social stress will be relatively short.

In mammals, opioids can inhibit the release of LH from the anterior pituitary by acting indirectly to inhibit the release of LHRH from the median eminence (see Bicknell (1985) for a review). Consequently exposure to stress or treatment with morphine during the period of LH release suppresses ovulation (e.g. Hulse *et al.*, 1982; Packmann & Rothchild, 1976). In birds, opioids also suppress LHRH release (Stansfield & Cunningham, 1987; Contijoch *et al.*, 1993) and so may inhibit spontaneous ovulation (Sakuari *et al.*, 1986). It is possible that stress could delay ovulation in hens by inhibiting LH release in an opioidergic manner. However, the pre-ovulatory peak of LH release is 4 h before oviposition (Furr *et al.*, 1973; Wilson & Sharp, 1973), yet stress did not start until 30 or 40 min before predicted oviposition time in the present experiments. Therefore it is unlikely that inhibition of LH release was the cause of the ovulation delays observed in the present experiments. It is not clear how stress might act to cause these delays.

That acute environmental stressors can act to disrupt ovulation time of hens has not been reported previously. As hens were killed in the present experiment, it is not known if an egg's oviposition is delayed as a consequence of its ovulation being delayed on the previous day (i.e. the time from ovulation to oviposition is not reduced), or if its oviposition is at the normal time (i.e. the time from ovulation to oviposition is shorter than normal). If oviposition time is normal, then the egg may spend less time in the uterus as a result of the delayed ovulation and this may reduce eggshell quality. It is also important to know what effect ovulation delays have on the timing of subsequent ovulations. The ovulations in an egg sequence occur at progressively later times in the day until the sequence ends with a pause day on which no egg is laid, and then the next sequence begins with ovulations occurring early in the day (Etches, 1990). If ovulations subsequent to the delayed ovulation are also delayed, then the positions of these eggs in the sequence may effectively be advanced and therefore the egg sequence may end sooner. If stress shortens egg sequences by delaying ovulation, then this would increase the proportion of days on which no egg was laid (pause days), and consequently the number of eggs laid per bird per day will

be reduced. Thus, the finding that acute stress can act to delay ovulation hens may be important in terms of egg numbers or eggshell quality.

To summarise, Experiment 1 provided no evidence to indicate that ovulation delays are caused by social stress. However, Experiment 2 indicated that social stress greatly reduced the proportion of hens which had ovulated before 35 min after predicted oviposition time, suggesting that ovulation delays were induced. Thus it is concluded that social stress can induce ovulation delays (Experiment 2) but that these delays may be relatively short, i.e. less than 1 h (Experiment 1). As this effect of stress on ovulation has not been reported previously, and it may have consequences for eggshell quality and egg numbers, it merits further investigation.

# Appendix 4: **Published Papers**

The following papers relating to experiments described in this thesis have been published.

**Reynard,M. & Savory,C.J. (1995).** Experiments on stress-induced delayed oviposition in hens. In: *Proceedings of the 19th International Congress of the International Society for Applied Ethology* (eds. Rutter,S.M., Rushen,J., Randle,H.D. & Eddison,J.C). pp.215-216. UFAW, U.K.

**Reynard,M. & Savory,C.J. (1995)**. Duration of stress-induced oviposition delays in hens. (abstract) British Poultry Science 36(5):863. Also in: *Proceedings of the World's Poultry Science Association United Kingdom Branch Spring Meeting*, Scarborough, 22-23 March 1995. pp.88-89.

**Reynard,M. & Savory,C.J. (1996)**. Social stress can delay both ovulation and oviposition in laying hens. In: *Proceedings of the World's Poultry Science Association United Kingdom Branch Spring Meeting*, Scarborough, 20-22 March 1996. (in press).

# Experiments on Stress-induced Delayed Oviposition in Hens

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## Introduction

Exposing laying hens to stressors such as change in group composition can induce oviposition delays (Hughes 1979). Although the physiological mechanisms underlying stress-induced oviposition delays are not known it is hypothesised that opioids released in response to stress inhibit the secretion of arginine vasotocin, the neurohypophyseal hormone responsible for promoting uterine contractions. This is analogous to the disruption of parturition and lactation in mammals due to stress-related opioidergic inhibition of oxytocin release (Leng & Russell 1989).

The aim of these experiments was to investigate how the duration of oviposition delay is related to the timing of stress (Experiment 1), and to investigate the role of opioids in this response through the use of an antagonist (Experiment 2) and an agonist (Experiment 3).

## Experiment 1

A pilot experiment suggested that periods of social stress of up to 6 h can induce oviposition delays, with some birds not laying during this disturbance. These birds were more likely to lay shortly after the end of the stress period when their ovipositions were less than 130 min overdue. This, along with the range of oviposition delays for eggs laid during stress periods, led to the hypothesis that hens become less able to lay their eggs after delays of more than 130 min, even if the stress is removed. The aim of this experiment was to test this hypothesis by exposing hens to periods of social stress of various duration, starting 1 h before expected oviposition.

ISA-Brown laying hens (n=47), 26 weeks old, were housed individually in cages while oviposition times were recorded automatically. When a hen's next oviposition time could be predicted from the egg laying records it was exposed to a period of social stress (relocation to another cage containing 3 unfamiliar hens). Each bird was exposed to one period of social stress which began 1 h before its predicted oviposition time and lasted 0.3-6 h.

From the egg laying records on adjacent days, an expected oviposition time for the day of treatment was calculated. The difference between this and the observed oviposition time, the delay, was calculated. Nineteen birds did not lay during their period of stress. These hens were significantly less likely to lay shortly after (<1 h) the stress ending if oviposition was already more than 130 min overdue (Fisher exact test, $p<0.05$). The mean oviposition delay for 5 eggs not laid shortly after the stress ending was 11.0 h. The range of oviposition delays (max. = 168 min) for eggs laid during stress also supports the hypothesis that birds become less able to lay following a delay of around 2-3 h, and then retain their eggs for much longer.

## Experiment 2

This experiment investigated the effect of the non-specific opioid antagonist nalmefene on the duration of stress-induced oviposition delays.

From 36 ISA-Brown hens, 27 weeks old and housed as before, 18 birds with predictable oviposition times were selected and randomly allocated to receive an intravenous injection of either nalmefene (0.5 mg/kg, n=9) or saline (0.5 ml/kg, n=9). Each bird was injected 30 min before its predicted oviposition time and then exposed to stress by housing it in a new cage with 5 unfamiliar hens. Oviposition times during stress were recorded and their delays calculated as before.

Oviposition delays were significantly reduced by treatment with nalmefene compared to saline ($p<0.01$, by two-tailed Mann-Whitney test, See Fig. 1).

## Experiment 3

The aim of this experiment was to investigate the effect of the selective kappa agonist U50488 on oviposition time. Previous tests with morphine, a mu receptor agonist, were inconclusive.

From 35 ISA-Brown hens, 47 weeks old and housed as before, 22 birds with predictable oviposition times were selected and randomly allocated to receive an intravenous injection of either U50488 (4 mg/kg, n=10) or saline (0.9 ml/kg, n=12). Birds were injected 30 min before their predicted oviposition times then left undisturbed in their usual cages. Oviposition times following the injections were recorded and their delays calculated as before.

Ovipositions of the U50488 treated birds were delayed significantly longer than those receiving saline ($p<0.05$, by two-tailed Mann-Whitney test, See Fig. 2).

## Conclusions

Stress-induced oviposition delays fall into two categories, long-term and short-term (<3h), with the timing of stress relative to the expected oviposition time influencing which of these occurs.

The non-specific opioid antagonist nalmefene reduced the duration of oviposition delay in response to social stress. The selective kappa opioid agonist U50488 induced oviposition delays in unstressed birds. Both these findings support the hypothesis that endogenous opioids are involved in mechanisms underlying stress-induced oviposition delays.

**Fig. 1**

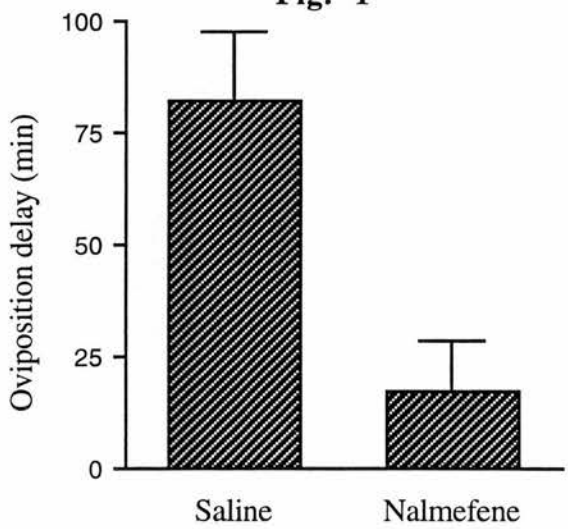


Fig. 1: Duration of oviposition delay in stressed hens treated with 0.5 mg/kg nalmefene (mean + SEM, n=9) or 0.5 ml/kg saline (mean + SEM, n=9).

**Fig. 2**

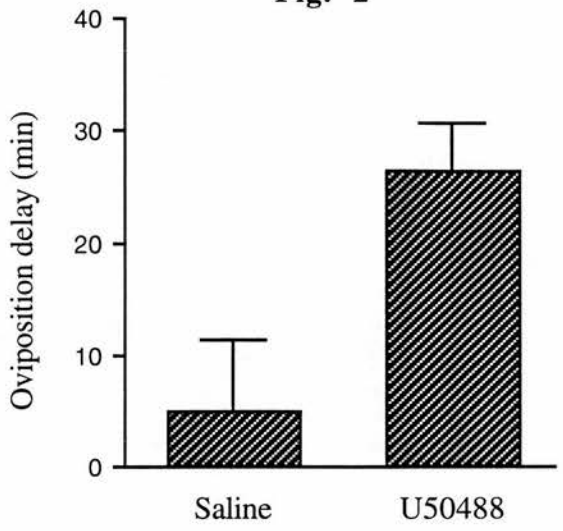


Fig. 2: Duration of oviposition delay in unstressed hens treated with 4 mg/kg U50488 (mean + SEM, n=9) or 0.9 ml/kg saline (mean + SEM, n=11).

**Acknowledgement**

The financial support of the British Egg Marketing Board (MR) is gratefuly acknowledged.

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Leng,G. & Russell,J.A., 1989. Opioids, oxytocin and parturition. In: Dyer,R.G. & Bicknell,R.J. (Eds.): Brain Opioid Systems in Reproduction. Oxford University Press, 231-256.

# Duration of Stress-Induced Oviposition Delays in Hens

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It is known that environmental stressors such as change in group composition, relocation, or removal of access to regularly used nest boxes can cause hens to delay oviposition, and that this can lead to characteristic eggshell abnormalities such as white banding, slab-sides and dusting (Hughes, 1979; Hughes *et al., 1986*). As the type of eggshell defect may be related to the duration of oviposition delay, this study was carried out to investigate the effect of the timing of a period of social stress on the duration of egg over-retention.

It was found that exposing birds to social stress (relocation from individual cages to larger cages with 3 unfamiliar companions) for periods of 4-5 h spanning the expected oviposition time potently induced oviposition delays, with many birds retaining their eggs until they were returned to their home cages. In these birds, oviposition occurred soon after (8-22 min) the end of the stress period only when the egg was still less than about 3 h overdue. In contrast, when the stress period did not end until after this 3 h-threshold, the egg was not laid until much later, after a delay of 10-19 h. Thus the durations of oviposition delays depend on the timing of the stress, and can be classified as being either short- or long-term.

When they were eventually laid all of the eggs delayed long-term carried a white band of extraneous calcification, while the subsequent egg was slab-sided in each case (because of contact with the delayed egg in the uterus) and was often laid prematurely with a soft or thin shell. Short-term oviposition delays typically resulted in the eggshells acquiring a superficial layer of calcium carbonate on top of the cuticle, visible as a white chalky coating or dusting on the surface of brown eggs.

As a long-term oviposition delay results in a banded and a slab-sided egg which are both downgraded, while a short-term delay causes only minor eggshell dusting, the 3 h-threshold may be a commercially important finding.

HUGHES,B.O., GILBERT,A.B., & BROWN,M.F.(1986) Categorisation and causes of abnormal egg shells: relationship with stress. *British Poultry Science*, **27:** 325-337.

HUGHES,B.O. (1979) Aggressive behaviour and its relation to oviposition in the domestic fowl. *Applied Animal Ethology*, **5:** 85-93.

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# Social Stress can Delay both Ovulation and Oviposition in Laying Hens

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Oviposition is usually followed by ovulation of the subsequent egg 15-45 min later, and both processes are associated with ovarian hormonal changes (Bahr & Johnson, 1991). A variety of environmental stressors can cause hens to delay oviposition (e.g. Hughes *et al.*, 1986). The aim of this study was to see if the subsequent egg's ovulation is also delayed. Social stress (relocation of hens from individual battery cages to a 0.6 $m^2$ holding pen where group size was 6-11) was used to induce oviposition delays.

The first experiment compared the position of the ovum at normal and delayed oviposition times. ISA Brown hens whose next oviposition times could be predicted were randomly allocated to either control (n = 11) or social stress (n = 12) treatments. Social stress started 30 min before predicted oviposition time. Hens from both treatments were killed by sodium pentobarbitone injection immediately after oviposition (or 4 h after predicted time if oviposition did not occur), and their oviducts examined. Ovipositions were delayed significantly longer (Mann-Whitney, W=83, $P<0.01$) by stress treatment (median = 104 min) than by control treatment (median = 4 min). Ovulation was significantly more likely to occur before oviposition in hens exposed to stress (Fishers exact test, $P<0.01$). From the positions of the subsequent eggs in the oviduct and their stages of development it was concluded any ovulation delays could not have exceeded 1 h.

The second experiment examined the effects of control and stress treatments on ovulation 35 min after predicted oviposition time; this should indicate whether stress can delay ovulation more precisely than Experiment 1. ISA Brown hens whose next oviposition times could be predicted were randomly allocated to either control (n = 26) or social stress (n = 27) treatments. Stress started 40 min before predicted oviposition time, and all hens were killed 35 min after predicted oviposition time. Compared with the control treatment, both oviposition ($X^2$=45.3, 1df, $p<0.001$) and ovulation ($X^2$=16.2, 1df, $p<0.001$) were significantly less likely to have occurred before death with the stress treatment (see Table).

From the second experiment it was concluded that social stress can delay both oviposition and ovulation. From the first experiment there was no evidence of ovulation delays exceeding 1 h.

| | Control | Social stress |
|---|---|---|
| Sample size | 26 | 27 |
| Hens laying | 25 (96%) | 1 (4%) |
| Hens ovulating | 18 (69%) | 4 (15%) |

Table. Effects of control and social stress treatments on the number of hens laying and the number of hens ovulating before 35 min after predicted oviposition time.

BAHR,J.M. & JOHNSON,P.A. (1991) Reproduction in poultry. In *Reproduction in Domestic Animals*, 4th edn. (ed. Cupps,P.T.), pp.555-575, Academic Press Inc.

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